CAVEOLIN-1 RESTORES PANCREATIC CANCER CELL DIFFERENTIATION AND MEMBRANOUS E-CADHERIN THROUGH SUPPRESSION OF EPITHELIAL-MESENCHYMAL TRANSITION

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Dedicated to my parents, my sister, & Heba.

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2. Abstract

Il tumore al pancreas è un cancro che origina dalle cellule all'interno della ghiandola addominale chiamata pancreas. La prognosi è considerata generalmente grave a causa della diagnosi spesso tardiva. Il cancro al pancreas non presenta all'inizio chiari sintomi ed e' caratterizzato da una rapida disseminazione metastatica e resistenza alla chemioterapia. Recentemente queste due caratteristiche sono state descritte come conseguenza di un processo di transizione epitelio-mesenchimale (EMT) delle cellule tumorali.

In questo studio abbiamo cercato di chiarire il ruolo della proteina di membrana caveolina-1 (CAV-1) in EMT. Caveolina-1 è la molecola principale costituente delle caveolae, invaginazioni ricche di sfingolipidi e colesterolo della membrana plasmatica. E' stato riportato che Cav-1 è implicata nella regolazione dell'espressione della proteina E-caderina e nel processo EMT. Noi abbiamo infettato una linea cellulare umana tumorale pancreatica, Panc 10.05, con il vettore retrovirale pBABE con inserito o meno il c-DNA della caveolina-1. Il risultato e' stato che le cellule Panc10/cav-1 presentavano una morfologia poligonale ed erano

più aderenti tra loro rispetto alle cellule trasfettate con il vettore vuoto (Panc10/pBabe) che mostravano una morfologia fusiforme e allungata e meno contatto tra le cellule. Inoltre, l'analisi con immunofluorescenza e western blot ha dimostrato che nelle cellule Panc10/cav-1 era ripristinata l'espressione della proteine E-caderina e β-catenina a livello della membrana plasmatica. La perdita dell'espressione di E-caderina è considerata un marker del processo di EMT e il suo recupero è una prova convincente dell'inibizione di EMT. Per approfondire il nostro studio abbiamo analizzato l'espressione di proteine regolatrici della E-caderina. Uno degli inibitori, Snail, è risultato essere down-regolato nelle cellule Panc10/cav-1. Abbiamo anche mostrato che l'espressione della cav-1 attenuava l'attivazione di numerose molecole coinvolte in EMT, come ERK, Smad2 e AKT. La disattivazione di queste molecole si traduceva nella diminuzione della migrazione e invasione delle cellule tumorali oltre a diminuire la resistenza alla chemioterapia. Panc10/cav-1 coltivate su matrigel, sistema di coltura tridimensionale, ha mostrato la formazione di sfere regolari, poche ramificazione e irregolarità che sono un'indicazione di EMT e invasività. Inoltre abbiamo mostrato che la linea cellulare tumorale pancreatica altamente metastatica, ASPC-1, quando incubata con il peptide di CAV-1, produceva un significativo arresto del ciclo

cellulare. Infine, abbiamo iniettato le cellule Panc10 nel fianco di topi nudi, cosiddetti perche' atimici e quindi immunodepressi. I tumori ottenuti dall'iniezione di Panc10 /cav-1 erano più piccoli in peso e in volume rispetto ai tumori ottenuti con le cellule Panc10/pBabe. Sorprendentemente i tumori Panc10/cav-1 presentavano agglomerati di cellule differenziate e ben organizzate che erano assenti nei tumori Panc10/pBabe. Queste cellule differenziate esprimevano E-caderina e β-catenina a livello della membrana cellulare oltre ad esprimere a basso livello la proteina Snail.

In conclusione questi risultati suggeriscono che la cav-1 agisce come inibitore del processo EMT nelle cellule tumorali pancreatiche attraverso il ripristino della proteina E-caderina e l'inibizione di molecole regolatrici di EMT. CAV-1 puo' rappresentare potenzialmente una cura per la malattia del cancro al pancreas.

Pancreatic cancer is considered to be one of the deadliest cancers due to its rapid metastasis and high chemoresistance. Recently, these two characteristics were identified to be a consequence of epithelial to mesenchymal transition (EMT). We wanted in this study to elucidate caveolin-1 (cav-1) role in EMT. Caveolin-1 (cav-1) is the main constituent molecule of caveolae, which are omega (Ω)-shaped invaginations found in plasma membrane. Cav-1 has been found to be implicated in regulation of E-cadherin and EMT, however little is known about the regulation mechanism. In this study, we infected pancreatic cancer cell line, Panc 10.05, with cav-1 expressing vector (Panc10/cav-1). Consequently to cav-1 infection, Panc10/cav-1 cell line displayed polygonal morphology and more cell adherens compared to Panc 10.05 infected with empty vector (Panc10/pBabe), which displayed spindle-shape morphology and less cell-cell contact. Also, our immunoblotting and immunofluorescence analysis showed that Panc10/cav-1 restored E-cadherin and β-catenin expression in the plasma membrane. E-cadherin loss is conceded as the hallmark of EMT, and its restoration is a convincing evidence of EMT inhibition. To study how cav-1 restored E-cadherin expression, we performed immunoblotting analysis for E-cadherin expression regulators. Snail ,is one of E-cadherin inhibitors, was found to be down-regulated in

Panc10/cav-1. Also, we found that cav-1 attenuated the activity of several molecules in EMT pathway such ERK, Smad2 and AKT in Panc10/cav-1 cells. This attenuation of EMT molecules activity was accompanied by decrease in migration, invasion and chemoresistance. Also, Panc10/cav-1 grown on three-dimensional culture showed regular spheres, and less branching and irregularity which are an indication of EMT and invasiveness. Furthermore, we showed that the metastatic pancreatic cancer cell line, Aspc-1, when incubated with cav-1 peptide, displayed significant cell cycle arrest. Finally, we injected infected cell in nude mice flank. Panc10/cav-1 tumors were smaller in weight and volume compared to Panc10/pBabe tumors. Astonishingly, Panc10/cav-1 tumors displayed nests of differentiated arranged cells surrounded by poor differentiated cells which were absent in Panc10/pBabe tumors. These differentiated cells expressed E-cadherin and β-catenin in the cell membrane, and down-regulated Snail expression.

In summary, these results provide evidence that cav-1 act as an EMT inhibitor in pancreatic cancer cells, through restoration of E-cadherin expression and inhibition of EMT initiator molecules. Also, cav-1 appears to be an important regulator of invasion,

chemoresistance, tumor proliferation and cell differentiation which presents cav-1 as a potential cure for pancreatic cancer disease.

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3. Introduction

3.1 Pancreatic Cancer

3.1.1 Epidemiology

Pancreatic ductal adenocarcinoma (PDA) is the fourth most common cause of cancer-related death and its incidence is estimated to be around 9 patients per 100,000 individuals (Jemal et al., 2009). With a five-year survival rate of only 3% and a median survival of less than 6 months, a diagnosis of pancreatic cancer carries one of the most dismal prognoses in all of medicine. Patients in their 70's or 80's experience unexplained weight lose, back pain , altered bowel syndrome habit, or sudden onset of jaundice. During clinical evaluation, a mass is found in the pancreas, usually the head of the pancreas, the right sided-region of pancreas adjacent to the duodenum. Due to the late diagnosis of the disease, in 80% of the patients is clinically evident that the cancer has already spread to other organs, most commonly to the liver (Kern, Hruban, Hidalgo, & Yeo, 2002). The other 20% of patients who have no detectable metastatic disease are candidates for surgical resection. Patients with resected pancreatic tumor have between 15-20% to live five-year (Sohn et al., 2000). Even if surgical resection is performed, unfortunately, all pancreatic cancer

patients succumb to metastatic disease (Yachida & Iacobuzio-Donahue, 2009).

3.1.2 Risk Factors

Due to incurability of pancreatic cancer patients, many researchers have inspected disease prevention and risk factors identification and involvement in development of PDA. A number of risk factors have been identified (Lowenfels & Maisonneuve, 2006). PDA is considered as a disease of elderly people. Pancreatic cancer is infrequent before the age 40, and the median age at diagnosis is 73 years (http://seer.cancer.gov/). Also, the Surveillance, Epidemiology and End Results (SEER) based on the U.S population shows that black African population has higher risk of development of PDA more than white population. Silverman et al showed that this higher incidence of PDA in black African is due to environmental factors like elevated body mass index and higher alcohol consumption and when these factors are absent, incident rate of PDA in black African probably will not exceed incident rate of white Americans (Silverman et al., 2003). On the contrary, Pernick et al assumed that genetic mutations and biomarkers

differences between the two races are the explanation of higher incidence rate in black African more than white cucasians (Pernick et al., 2003).

As smoking cigarettes is the most documented etiologic factor, PDA can be preventable by stopping this habit(Stolzenberg-Solomon et al., 2001; Villeneuve, Johnson, Hanley, & Mao, 2000) .Cigarettes smoking nearly doubles the risk of PDA, and it is believed that as one in four cases of PDA may be attributable to smoking (Lowenfels & Maisonneuve, 2006). Other established risk factor include high fat and meat diets, low serum folate levels, obesity, diabetes mellitus, and chronic pancreatitis (Everhart & Wright, 1995; Lowenfels & Maisonneuve, 2006; Michaud et al., 2001; Michaud et al., 2002). As coffee was accused to be one of the risk factors of the PDA, more recent studies do not show any significant association (Lowenfels & Maisonneuve, 2006).

One of the interesting risk factor of PDA is family pancreatic cancer history. It is considered to cause pancreas cancer in less than 10% of all PDA cases(Bardeesy & DePinho, 2002; Maitra &

Hruban, 2008). Amundadottir et al analyzed 32,534 cancer cases diagnosed in Iceland from 1 January 1955 till December 31 2002. They evaluated any significance of the familial clustering for each relationship separately, all relationships combined (first- to fifth-degree relatives) and for close (first- and second-degree) and distant (third- to fifth-degree) relatives. They found that pancreas cancer is increased by 2.3-fold for first-degree relative, and folds decreases with the distance of the relative until it reaches 0.9-fold in fifth-degree relative (Amundadottir et al., 2004). Also, Klein et al. have demonstrated that individuals with a strong family history of PDA have a significantly increased risk of developing pancreatic cancer (Klein et al., 2004). Table 1 summarizes some of germ-line alteration which can be associated to risk pancreas cancer (Bartsch et al., 2002; de vos tot Nederveen Cappel et al., 2003; Giardiello et al., 2000; Goggins et al., 1998; Goggins et al., 1996; Lowenfels et al., 1997; Lynch et al., 2005; Ozcelik et al., 1997; Petersen et al., 2006; Su et al., 1999).

Maitra et al had revealed six characteristic of the genetic syndromes associated with the familial clustering of PDA (Maitra & Hruban, 2008). Firstly, they mentioned that cancer is not inevitable

in mutation gene carries. This appears in Goggins et al study which they screened 41 adenocarcinoma of pancreas (30 pancreatic adenocarcinoma xenografts and 11 PDA cell lines) and four (9.8%) of the 41 patients harbored a germ-line *BRCA2* gene mutation (Goggins et al., 1996). One of the four mutation cases had a breast cancer-relative. Obviously, strong family history of cancer can be used for better screening of gene mutation carriers.

Secondly, patients identified with these gene mutations, should be screened and advised that they may acquire a PDA in the future. For example, Peutz-Jeghers syndrome is characterized by hamartomatous gastrointestinal polyps and mucocutaneous pigmentation, and melanocytic macules on the lips and buccal mucosa (Giardiello et al., 2000). Patients with these syndrome should be tested for *STK11/LKB1* mutation. Those who carry this mutation will be screened for asymptomatic PDA (Canto et al., 2006).

Thirdly, most of pancreatic gene mutations are associated with developing other cancers, too. For instance, *p16/CDKN2A*

mutation carriers have a risk of developing melanoma that can reach 28% at the age of 80 (Begg et al., 2005) .

Fourthly, PDA with microsatellite instability often shows a distinct medullary histology. These medullary carcinoma hallmarked with DNA mismatch repair defects in *MHL1/MSH2* genes and demonstrates poor cell boundaries and syncytial growth pattern of the cells (Wilentz, Goggins et al., 2000). PDA patients exhibit this morphologic appearance may benefit from genetic counseling.

Fifthly, different therapeutic regimes can be selected according to patients' genetic mutations. For example, the protein product of *BRCA2* gene binds with the protein products of the Fanconi anemia complementation genes (the *FANC* genes) to form a homologues recombination. Van der Heijden et al have demonstrated that PDA with *BRCA2* or other proximal FANC gene mutations are particularly sensitive to Mitomycin C and radiation therapies which produce DNA cross-linking then breaks in double strands (van der Heijden et al., 2004).

Lastly, some ethnic groups can carry some of these genetic mutations. This is implied in the Ashkenazi Jewish population that 1% of it carried the 6174delT *BRCA2* gene mutation (Ozcelik et al., 1997).

3.1.3 Pathology

Pancreatic cancer or pancreatic ductal adenocarcinoma (PDA) are tumors arising from pancreatic exocrine ductal cells. 70% of PDA originates from the head of the pancreas and the rest arises from the body and the tail of the pancreas. Other tumors arise from tissues surrounding pancreas like ampullary adenocarcinoma which arises from the ampulla of Vater, pre-ampullary adenocarcinoma which describes tumors near the Ampulla of Vater, distal cholangiocarcinoma which arises from the distal bile duct adjacent to the Ampulla and duodenal adenocarcinoma which arises from the duodenum adjacent to the Ampulla. There are other tumors arise from pancreas like neuroendocrine tumors. My thesis will focus only on PDA pathology.

Since there are few millimeters of separation between these tumors, it is essential to differentiate between them as they vary greatly of their prognosis. Yeo et al observed at 242 patients that underwent pancreaticoduodenal resection for periampullary adenocarcinoma over 22 years (Yeo et al., 1998). Of the 242 patients with resected periampullary adenocarcinoma, 149 (62%) were PDA, 46 (19%) were ampullary adenocarcinomas, 30 (12%) were cholangiocarcinomas, and 17 (7%) were duodenal cancers. The post-operative 5 year survival percent was lowest in PDA (15%), then ampullary adenocarcinoma (25%), cholangiocarcinomas (27%) and duodenal adenocarcinoma (59%). In this study, Yeo et al displayed differences between PDA and non PDA patients' outcome. Also, they indicated that factors like tumor differentiation, lymph nodes status and tumor resection margins are correlated with poor prognosis of PDA patients.

3.1.4 Pancreatic cancer and pancreatic cancer precursors

Pancreatic ductal adenocarcinoma -PDA- is considered as synonym of pancreatic cancer. At the gross level, PDA appears as firm sclerotic mass with poor defined borders. At the microscope

level, PDA cells are consisting of infiltrating gland-forming neoplastic epithelium surrounded by a strong desmoplastic reaction. The desmoplastic reaction is so usually strong that the neoplastic cells becomes a minority in the tumor. Infiltrative cells around neurons and vessels are dominantly recognized in resected tumors (R.H. Hruban, Klimstra, & Pitman, 2006). PDA cells show similar cuboidal phenotype, express ductal antigens and grow in tubular structure like pancreatic ductal cells which led investigators to predict that exocrine cells are the origin of PDA (R.H. Hruban, Klimstra, & Pitman, 2006). This theory was firstly proposed in 1976 by Cubilla and Fitzgerald (Cubilla & Fitzgerald, 1976). They studied 227 cases with pancreatic cancer and 100 autopsies from normal pancreas as a control. They observed that the occurrence of ductal papillary hyperplasia was three times more in pancreas cancers than in controls. Moreover, they found that the incidence of ductal papillary hyperplasia is 37% in PDA where it was 12% in control group. Also, they observed 20% with marked atypia and 18% with carcinoma in situ of the pancreas cancer cases, but these two features were absent in the control cases. Since then any pre-cancerous lesion in pancreatic ductal cells is considered as a precursor of pancreatic cancer. The pre-cancerous lesions are divided in pancreatic intraepithelial neoplasia (PanIN), intraductal

papillary mucinous neoplasm (IPMN), and mucinous cystic neoplasm (MCN).

PanIN lesions can be papillary or flat, and are composed of columnar to cuboidal cells with various amounts of mucin. Hruban et al. divided these lesions into three basic categories PanIN 1, 2, 3 and PanIN 1 was divide to A and B depending upon the degree of cytologic and architectural atypia (Figure.1) (R. H. Hruban et al., 2001; R. H. Hruban et al., 2004). Many researchers compared between genetic mutations in PDA and PanIN. Their studies showed a remarkable similarity of mutations depending on the PanIN degree, for example telomere shortening in PanIN1 (van Heek et al., 2002), overexpression of p53 in precancerous glands (Boschman, Stryker, Reddy, & Rao, 1994), inactivation of *BRCA2* in late stages of PanIN (Goggins, Hruban, & Kern, 2000), early loss of heterozygosity (LOH) of p16 (INK4) and late LOH of *DCP4/SMAD4* (Heinmoller et al., 2000), overexpression of EGFR and Her-2/neu (ERRB2) (Day et al., 1996), and *K-ras* activation mutation (Terhune, Phifer, Tosteson, & Longnecker, 1998) (Figure.2). As so far, no valid method of pancreatic screening has been discovered. Identification of global gene expression is

suggested to be used as a biomarker to facilitate diagnosis and therapy (Sipos, Frank, Gress, Hahn, & Kloppel, 2009).

Intraductal papillary mucinous neoplasm (IPMN) is another precursor of PDA. IPMN is defined as a mucin-producing epithelial neoplasm ranging from benign to invasive carcinoma. In 1984 IPMN was firstly described by Ohhashi *et al*. in Japan, then after eight years IPMN was recognized in North-America (Belyaev et al., 2008). IPMN is classified by WHO in many categories depending on the degree of the atypia architecture (Longnecker, Adler, & Hruban, 2000). IPMN consists of IPMN adenoma which show differentiated columnar cells displaying minor or no dysplasia; IPMN border-line which shows moderate dysplasia, moderate loss of polarity, nuclear crowding, nuclear enlargement, nuclear hyperchromatism, and pseudopapillary; and IPMN carcinoma (carcinoma in situ) that displays sever dysplasia, mucin diminishing, cribriform growth, budding to the lumen, loss of polarity, loss of differentiation, cellular and nuclear polymorphism, nuclear enlargement and presences of mitosis. IPMN can arise from the main pancreatic duct (it can be invasive), or from secondary ducts, or even both (Nakagohri, Kenmochi, Kainuma, Tokoro, & Asano,

1999; Terris et al., 2000). IPMN carcinoma is predominantly consisting of colloid (mucinous noncytic) carcinoma, and the rest consists mainly of conventional tubular adenocarcinoma (Adsay, 2002). IPMN shares many genetic abnormalities with PDA, for instance mutations of the KRAS2 (Z'Graggen et al., 1997), TP53/p53 (Islam et al., 2001; Sasaki et al., 2003) and CDKN2A/p16 genes (Biankin et al., 2002), rare loss of SMAD4 expression (Biankin et al., 2002; Sasaki et al., 2003) and loss of *STK11/LKB1* gene (Sahin et al., 2003). Fortunately, IPMN has better diagnosis than PDA for producing radiographically identifiable ductal dilatation (Canto et al., 2006) and better diagnosis with surgical treatment, (Kanazumi et al., 2001).

Finally, mucinous cystic neoplasm (MCN) which is also a mucin-producing epithelial neoplasm but harbor a distinctive ovarian-type stroma absent in IPMN (R.H. Hruban, Klimstra, & Pitman, 2006).

Although 20% of pancreatic cancer patients are susceptible for pancreaticoduodenectomy operation, resected pancreas

patients prognosis tends to deteriorate after the surgery. There are many factors that affect the prognosis outcome like tumor differentiation, tumor diameter, lymph nodes status, positive resection margins, surgery out with specialist centers (Birkmeyer et al., 1999; Lim, Chien, & Earle, 2003; Sohn et al., 2000; Sperti, Pasquali, Piccoli, & Pedrazzoli, 1997; Yeo et al., 1997). Lately, other factors like systemic inflammation presence and node ratio were proposed to predict prognosis and adjuvant therapy plan (Jamieson et al., 2005; Riediger et al., 2009).

3.1.5 Pancreatic cancer genetical progression

Pancreatic cancer is defined as a pathology of accumulated of inherited or somatic mutations of oncogenes activation or tumor suppressor genes inactivation. Thanking to advanced technology, several genetical mutations were identified. As mentioned above, PanIN lesions, which are considered as precursors of PDA, harbor many but not all PDA genetical mutation (R. H. Hruban, Wilentz, & Kern, 2000).

3.1.5.1 KRAS

KRAS is the most common mutated gene in pancreatic cancer occuring in 90% of pancreatic cancer cases (Caldas & Kern, 1995). *KRAS* is a protooncogene activated by a mutation which permits *KRAS* product to play a crucial role in a range of cellular functions, including proliferation, survival, and motility. The activation of KRAS protein initiates when growth factors bind to growth factor receptors which results a downstream signal transduction to activate KRAS. In the active state KRAS binds to guanosine triphosphate (GTP). Upon conversion of GTP to guanosine diphosphate (GDP) by guanosine triphosphatase-activating protein (GAP), KRAS is turned off. Activating mutation prevents GAP from hydrolyzing GTP causing accumulation of active KRAS-GTP. Most of *KRAS* mutations occur in codon 12, 13, and 61 (Bos, 1989; Caldas & Kern, 1995). *KRAS* mutation is one of the earliest mutation in pancreatic cancer progression. 36% of PanIN-1A cases, 44% of PanIN-1B of cases and 87% PanIN-2/3 cases demonstrate *KRAS* mutations (Maitra, Fukushima, Takaori, & Hruban, 2005). Researchers have found that different PanINs in the same tumor can display different mutations suggesting that there are separate clonal evolution of multiple pancreatic

neoplasms within the same tumor (Moskaluk, Hruban, & Kern, 1997). *KRAS* mutation has an important role in PDA progression which is proven through the high frequency of mutation in PDA patients and animal model studies which revealed that animals begin to develop pancreatic cancers when they carry a mutation in *KRAS* (Fleming, Shen, Holloway, Davis, & Brekken, 2005; Hingorani et al., 2003; Moskaluk, Hruban, & Kern, 1997). Beside the role of PDA developing, *KRAS* plays a major role in pancreatic cancer proliferation (Fleming, Shen, Holloway, Davis, & Brekken, 2005).

3.1.5.2 HER-2/neu

HER-2/neu is a member of the epidermal growth factor receptor family and is over expressed in 69% of PDA cases (Day et al., 1996). Day *et al.* evaluated the expression of HER-2/neu in different pancreatic lesions and found a correlation between the increasing HER-2/neu expression and the increasing of pancreatic duct atypia (Day et al., 1996).

3.1.5.3 p16

The *p16* tumor suppressor gene, which is also known as *INK4*, is the most inactivated gene in PDA, its loss seen in 95% of PDA cases (Caldas et al., 1994; Schutte et al., 1997). This inactivation leads to phosphorylation of many growth proteins which cause loss of cell cycle control and unchecked proliferation (Sohn & Yeo, 2000). *p16* inactivation results by different mechanisms, including homozygous deletion, intragenic mutation with loss of the second allele and epigenetic silencing by promoter methylation (Maitra, Kern, & Hruban, 2006).

3.1.5.4 TP53

TP53 is a tumor suppressor gene, which is inactivated in approximately 50%-75% of pancreatic cancer (Redston et al., 1994). The majority of the inactivation of *TP53* gene occurs through intragenic mutation with loss of the second allele. In normal non-stressful status p53 protein, expression is very low. However, when cells are exposed to DNA damage, hypoxia or other insults, p53 becomes activated and induces growth arrest and/or apoptosis by the induction or repression of specific genes. In pancreatic cancer

cell, loss of p53 function permits the cell to survive and proliferate regardless of damaged DNA, which leads to more accumulation of additional genetic mutations (Vogelstein & Kinzler, 2004).

3.1.5.5 DCP4/SMAD4

Deleted in pancreatic carcinoma 4 gene (*DPC4)*, which is also known as *SMAD4*, on chromosome 18q21 is inactivated in approximately 55% of PDA, either by homozygous deletion (30%) or by intragenic mutations and loss of the second allele (25%) (Hahn et al., 1996). Smad4 protein performs a crucial role in signaling through the transforming growth factor-β -TGF-β- pathway. The TGF-β pathways starts when the TGF-β protein adheres to TGF-β receptors. This provokes an intracellular cascade of phosphorylation and nuclear localization of the Smad transcription factors Smad 4 and Smad 2/3 complex. In normal condition the TGF-β pathway plays a growth-inhibitory role by controlling the expression of specific target genes, subsequently, deficiency of Smad4 in PDA cells allows the cancer cells to grow uncontrollably (Siegel & Massague, 2003). Loss of Smad4 expression occurs only at PanIN-3 lesions, whereas almost all

noninvasive lesions maintain its expression (Iacobuzio-Donahue, Klimstra et al., 2000; Iacobuzio-Donahue, Wilentz et al., 2000; Wilentz, Iacobuzio-Donahue et al., 2000).

3.1.5.6 BRCA2

Patients with inherited tumor suppression gene *BRCA2* mutation are identified to be in a risk of developing breast, ovarian, prostate, stomach and malignant melanomas tumors ("Cancer risks in BRCA2 mutation carriers. The Breast Cancer Linkage Consortium", 1999; Hamilton, 2009; Liede, Karlan, & Narod, 2004; Ostrander & Udler, 2008; Ramus & Gayther, 2009). Additionally, the same patients have a relative risk of 3.51 (95% CI 1.87-6.58) of developing pancreatic cancer ("Cancer risks in BRCA2 mutation carriers. The Breast Cancer Linkage Consortium", 1999). Goggins *et al* recognized mutation in *BRCA2* occurred late in the development of PDA, nevertheless this was a very small study of 14 PanIN lesions with a single mutation (Goggins, Hruban, & Kern, 2000). *BRCA2* mutation is considered to occur late in pancreatic cancer cells, which can tolerate DNA damage, as *BRCA2* loss

leads to lethal chromosomal abnormalities which is not tolerated in normal cells (Venkitaraman, 2002).

3.2 Caveolae and Caveolin

3.2.1 Background

The advent of caveolae research owes its inception to their morphological identification in 1953. By transmission electron microscopy, caveolae appear an structures resembling “little caves”, which are 50- 100-nm vesicular invagination of the plasma membrane (Figure.3) (Palade, 1953). Caveolin-1 (Cav-1 or VIP21) was the first family member identified, and was demonstrated as structural component of caveolae and trans-Golgi derived transport vesicles. Since then, caveolae/caveolin-related research has identified a family of three proteins, caveolin-1, -2, and -3, all of which serve as protein markers for caveolae. Caveolae are considered by many to be a subset of lipid rafts, these are highly-ordered microdomains residing within the plasma membrane dramatically enriched in cholesterol and sphingo-lipids (glycol-sphingolipids and sphingo-myelin) in comparison with the surrounding phospho-lipid bilayers (D. A. Brown & London, 1998; Galbiati, Razani, & Lisanti, 2001b; Simons & Toomre, 2000). However, the classification of caveolae as a subset of lipid rafts may be not be completely accurate, as some proteins are known to selectively localize to either lipid rafts or caveolae but not in both

(Liu, Ying, & Anderson, 1997). The particular biochemical properties of caveolae/lipid rafts are conferred by their lipid composition. This has enabled investigators to efficiently purify caveolae-enriched membrane fractions from cells and tissues by sucrose gradient ultracentrifugation due to i) their reduced density (high buoyancy) and ii) resistance to solubilization by mild non-ionic detergents (Triton X-100) at 4°C (Chang et al., 1994; Lisanti, Sargiacomo, & Scherer, 1999; Lisanti, Scherer, Tang, & Sargiacomo, 1994; Lisanti, Tang, Scherer, & Sargiacomo, 1995). The majority of caveolae in cells and tissues require only Cav-1 expression for their proper formation, whereas Cav-2 is not absolutely required (Figure.3) (Drab et al., 2001; Razani, Combs et al., 2002; Razani et al., 2001). On the other hand, caveolae that are found in skeletal muscle tissue and cardiac myocytes are mainly Cav-3 generated (Figure.3) (Galbiati, Engelman et al., 2001; Razani, Woodman, & Lisanti, 2002).

3.2.2 Gene organization and evolution history

The structural component of caveolae remained unknown for many decades until a 22 KDa protein was isolated as one of

several proteins that became tyrosine-phosphorylated in v-Src-transformed chicken embryo fibroblasts (Glenney & Zokas, 1989). this protein was later termed caveolin (now renamed caveolin-1; (Scherer et al., 1996)) since monoclonal antibodies directed against this protein decorated the cytoplasmic protein coat of caveolae. As a result, caveolin-1 was identified as the first true protein marker of caveolae.

Subsequent cloning of caveolin-1 cDNA revealed that it was identical to another protein, known as VIP21, which had been cloned almost simultaneously (Glenney, 1992; Kurzchalia et al., 1992). Interestingly, VIP21 was isolated as an integral membrane protein component of trans-Golgi derived transport vesicles in MDCK cells, suggesting that caveolin-1/VIP21 may also possess a role in molecular trafficking, as well as oncogenesis. Since then, caveolin-1 (Cav-1) has been found to be expressed ubiquitously, albeit diversely, with the highest levels in adipocytes, endothelial cells, fibroblasts, smooth muscle cells, and a variety of epithelial cells. In accordance with this observation, it has become clear that the cellular functions of caveolin-1 are dependent on the cellular context.

Since the cloning of the first caveolin-1 cDNA sequence from a chicken genomic library by Glenney and Soppet in 1992 (Glenney, 1992), numerous other caveolin-1 sequences have been obtained from a whole range of vertebrates, including humans, bovine, dog, mouse, *Xenopus,* and the Japanese pufferfish *Fugus rubripes* (Table.2). These cloning efforts also identified a caveolin gene family within invertebrates, specifically *C. elegans,* demonstrating the ubiquity of caveolins among higher-evolved organisms (Scheel, Srinivasan, Honnert, Henske, & Kurzchalia, 1999).

Caveolin-2 and caveolin-3 were identified in 1996 through different experimental methods. Caveolin-2 (Cav-2) was discovered by micro-sequencing of a 20 KDa protein that co-purified with adipocytes-derived caveolae membranes (Scherer et al., 1996). Further characterization revealed that cav-2 co-localizes with cav-1 in caveolae, forms hetero-oligomers with cav-1, is co-expressed in many of the same cells and tissues, and requires cav-1 for proper transport from the Golgi apparatus to the plasma membrane (Parolini et al., 1999; Scherer et al., 1997). Caveolin-3 (Cav-3) was identified through data-base searches and traditional cDNA library

screening in an attempt to find cav-1 homologous (Tang et al., 1996). Cav-3 (also known as M-caveolin) is an interesting member of the caveolin gene family, as it is expressed predominantly in striated muscle cells (Song, Scherer et al., 1996).

The three mammalian genes encoding members of the caveolin family share significant homology. The cav-2 protein is approximately 38% identical and 58 similar to cav-1, while cav-3 is more closely related to cav-1, with 56% identity and 85% similarity (Table.3). Interestingly, however, cav-1 and cav-2 are located in very close proximity to each other on human chromosome 7q31.1, while cav-3 is located on a different chromosome (3p25). Cav-1 is located ~ 19 kb downstream of cav-2 on chromosome 7q31.1, while cav-2, in turn is located ~67 kb downstream from the D7S552 microsatellite locus (Engelman, Zhang, Razani, Pestell, & Lisanti, 1999). Intriguingly, this D7S552 microsatellite repeat marks a region commonly deleted in human cancers (Engelman, Zhang, Galbiati, & Lisanti, 1998). This genetic observation implicated cav-1 as a potential tumor suppressor, when taken together with the other functional data showing that cav-1 possesses transformation-suppressor activity in cultured cells (Koleske, Baltimore, & Lisanti,

1995). Deletion or mutation of the cav-3 gene on chromosome 3p25 is also associated with a human disease, namely autosomal dominant Limb-girdle Muscular Dystrophy, Type-1C (LGMD-1C) (Minetti et al., 1998).

The phylo-genetic relationship among caveolin family members is shown using the Clustal-W program, with the *C. elegans* cav-1 sequence located the furthest in terms of predicted evolutionary distance (Figure.4). While the evolutionary history of the caveolin genes has not been clearly defined, there are clues present within the sequence and genomic organization that suggest possible mechanisms for the origin of the individual genes. One interesting observation made from *C. elegans* is that while *C. elegans* cav-1 contained two exons, the region that is homologues to mammalian caveolin is encoded by only single exon, suggesting that mammalian caveolins are derived from this particular exon (Tang et al., 1997). Another point is that the overall genomic organization of the cav-1 and cav-2 is relatively conserved from humans to pufferfish. However, there is a significantly higher degree of homology between human cav-1 and *Fugu* cav-1, when compared to human cav-2 and *Fugu* cav-2. This may suggest that

cav-2 can tolerate more sequence diversity (a higher mutation rate), despite being located at the same genetic locus (Engelman, Zhang, & Lisanti, 1999). Finally, two observation extracted from the human genomic sequence insinuate that some family members may have arisen through gene duplication events: i) the exon-intron boundaries in the last exons of cav-1 and cav-2 are analogues; ii) exon 2 of cav-2 is divided into two parts (named 2a and 2b) by an intron whereas the two homologues portions in the cav-1 and cav-3 sequences are fused together to form the final exon(Engelman, Zhang, & Lisanti, 1998, , 1999). this second point suggests that cav-2 may be the precursor for cav-1 and cav-3 (Williams & Lisanti, 2004).

3.2.3 Characteristics structural features

While research has begun to provide insights into the genetics of caveolins, the structural features of this protein family are poorly defined. Currently, there exists no crystallographic data that could suggest a three dimensional structure of any caveolin family member. However, information gleaned from the protein sequence has allowed predictions of structure and motifs within the

protein. For instance, all three caveolins possess an invariant “FEDVIAEP” stretch within their hydrophilic N-term domains that has come to be termed the “caveolin signature motif” (Scherer et al., 1996; Tang et al., 1996). However, the importance of this sequence or motif has yet to be determined. The majority of the remaining structure-related research has focused on cav-1 as the proto-typical family member.

Through a variety of experimental methods, it has been determined that the major sub-cellular location of cav-1 is at the plasma membrane. Based on a combination of primary sequence (hydrophilicity plots) and mutational analysis, cav-1 is predicted to have a membrane-spanning hairpin-like structure, with both N- and C-terminus directed intracellularly towards the cytoplasm (Figure.5). This typical membrane spanning model is supported by the findings that i) antibodies directed against the cav-1 N- or C-terminus require permeabilization of cells in order to bind cav-1, ii) cell surface biotylation does not label cav-1, and that there are well-known palmitoylation and tyrosine phosphorylation sites within both N- and C-terminal domains of the protein (Dietzen, Hastings, & Lublin, 1995; H. Lee et al., 2000; S. Li, Seitz, & Lisanti, 1996;

Sargiacomo et al., 1995). Palmitoylation and tyrosine phosphorylation are both cytoplasmically generated post-translational modifications.

Among caveolins, the predicted domains virtually span the same number of residues. The N-terminal domain comprises the first 70-101 residues (cav-1= 101 residues, cav-2/cav3= 70-74 residues), with the putative transmembrane domain occupying 32 amino acids, and the C-terminal domain 42-44 amino acids. Interestingly, two cav-1 isoforms (α and β) have been identified, with the β-isoform arising from an internal translational start site that generates a shorter cav-1 isoform, with a 31 residue truncated N-terminus (Scherer et al., 1995).

Generally speaking caveolins are small proteins (18-24 KDa). Structurally, perhaps one of the most interesting and significant findings about cav-1 is that it forms an oligomeric complex comprised of approximately 14 to 16 monomers. The discovery that cav-1 exists as a high molecular mass complex was achieved through velocity gradient ultracentrifugation. By this assay

system, cav-1 was found to migrate as a 350-400 KDa complex, composed exclusively of cav-1 (Sargiacomo et al., 1995). In addition, experiments with cav-1 deletion mutants mapped this "oligomerization domain" to residues 61-101 (Figure.6). Interestingly, also cav-3 forms large ~350-400 KDa oligomeric complexes *in vivo,* while cav-2 requires cav-1 to participate in the formation of these higher molecular mass complexes.

Moreover, cav-1 contains a region within the oligomerization domain that is presumed to be the main functional domain of caveolin family members. This region, spanning cav-1 amino acid positions 82-101 (Figure.6), has been termed "the caveolin-scaffolding domain" (CSD) since it mediates binding of a number of signaling proteins (S. Li, Couet, & Lisanti, 1996; Scherer et al., 1997). The CSD behaves as a modular protein domain, akin to SH2/SH3 and WW domains, and allows the binding of multiple classes of signaling molecules, including Src-family tyrosine kinase, eNOS, receptor tyrosine kinases (such as Insulin-R, EGFR, ErbB2/Neu, PDGFR), H-Ras, PKC isoforms, and PKA (Razani, Woodman, & Lisanti, 2002; Scherer et al., 1997). Aside from acting as a scaffolding protein to compartmentalize these signaling

molecules, this region of cav-1 appears to inhibit the down-stream activation and signaling of many of these proteins (Razani, Woodman, & Lisanti, 2002). Screening of random peptide libraries using the caveolin-scaffolding domain receptor, has identified caveolin-binding-motifs on other proteins that mediate their high affinity association with the CSD (Couet, Li, Okamoto, Ikezu, & Lisanti, 1997). These caveolin-binding motifs consist of a series of 3-or-4 aromatic amino acids (Tryptophane –W-, phenylalanine –F-, and tyrosine –Y-), with a particular spacing.

3.2.4 Localization and Function

Beginning with its early characterization, cav-1 was shown to localize to plasma membrane caveolae, demonstrating a punctate staining pattern at the margins of cells (Glenney, 1992). Additionally, Simons *et al* have shown positive staining of the Golgi apparatus and trans-Golgi-derived transport vesicles (Kurzchalia et al., 1992). Since then, recent research has built upon the notion that caveolin localization is limited to membranes and vesicles by providing new evidence that cav-1 also exists in a soluble cytoplasmic form, as wells as a secreted form, depending on the

cell type (Liu, Rudick, & Anderson, 2002). While there are no N-terminal signal sequences in cav-1, the finding that the β-isoforms of cav-1 is targeted to the cytosol in skeletal muscle cells but hint at the importance of the first 31 amino acids in selectively targeting cav-1 to different cellular compartments (W. P. Li, Liu, Pilcher, & Anderson, 2001).

Cav-1 and cav-2 are tightly co-expressed in a diverse range of cells and tissues, suggesting that both utilize identical transcription regulatory pathways. Interestingly, cav-1 is required for proper membrane localization of cav-2. For example, when cav-2 is expressed in the absence of cav-1, cav-2 retained in the Golgi complex and rapidly degraded (Parolini et al., 1999). A mutant form of cav-1 found in up to 16% of human breast cancers, Cav-1 (P132L), has a single-amino acid substitution in the membrane-spanning region (Hayashi et al., 2001). Therefore, does not properly localize to the plasma membrane (H. Lee et al., 2002). Furthermore, cav-1 (P132L) behaves in a dominant-negative manner, causing the mis-localization and intracellular retention of wild-type cav-1 (H. Lee et al., 2002). Intriguingly, an analogous P-to-L mutation at position 104 within cav-3 has been detected in

patients with LGMD-1c. This mutation also behaves in a dominant-negative fashion (Galbiati, Razani, & Lisanti, 2001a; Galbiati, Volonte, Minetti, Bregman, & Lisanti, 2000; Minetti et al., 1998; Parolini et al., 1999). Wild type cav-3 also localizes to caveolae and the plasma membrane, but its tissue-specific expression in muscle cells led to the observation that cav-3 also associates with the T-tubule network (Minetti et al., 2002). In fact, cav-3 (-/-) deficient mice develop abnormalities in the organization of the T-tubule system, and clearly lack sarcolemmal caveolae in skeletal muscle fibs and cardiac myocytes (Galbiati, Engelman et al., 2001).

The upsurge in caveolin-related research has helped to clarify the existing purported functions of caveolae and identify new functions of these structures and their associated proteins. It is becoming clear that different caveolin proteins may have distinct role in certain cells types and tissues that are completely unrelated to their roles in other cells. The following are brief description of some of the functions of caveolins.

3.2.5 Vesicular transport

Numerous lines of research have implicated caveolae in the processes of vesicular trafficking e.g., transcytosis, endocytosis, and potocytosis. Their profuse appearance on both apical and basolateral faces of endothelial cells first suggested a role for caveolae in the process of transcytosis. Since that time, endothelial caveolae have now been shown to possess the molecular components utilized by other transport vesicles, during the processes of vesicle formation, docking and fusion, including vSNARE VAMP-2, GTPases, NSF and SNAP (S. W. Lee, Reimer, Oh, Campbell, & Schnitzer, 1998). In addition, dynamin localizes to caveolae and is concentrated at their caveolar necks, suggesting that dynamin is responsible for caveolae fission and the formation of intracellular caveolar vesicles, a.k.a, plasmalemmal vesivles (Henley, Krueger, Oswald, & McNiven, 1998). In terms of endocytosis, it appears that certain ligands and extracellular molecules such as cholera and tetanus toxins, are transported through caveolae, rather than via clathrin-dependent mechanisms (Montesano, Roth, Robert, & Orci, 1982). Finally, micro-organisms appear to have evolved mechanisms to gain cellar entry through caveolae, including SV40 and certain strains of *E. coli* (Norkin,

2001). For additional information on this topic, the reader is referred to a more detailed review of caveolae and endocytosis (Pelkmans & Helenius, 2002).

3.2.6 Cholesterol homeostasis

Research clearly demonstrates an intimate relationship between cholesterol, caveolin proteins, and caveolae organelles. Cav-1 is one of the few proteins that tightly binds cholesterol, in a very specific fashion (Murata et al., 1995). In addition, free cholesterol is required for proper caveolae formation, as brief treatment of cells with cholesterol-binding agents, such as nystatin and filipin, disrupt caveolae structure and function (Smart et al., 1999). In addition, the cav-1 promoter (Engelman, Zhang, Razani, Pestell, & Lisanti, 1999) contains two sterol regulatory elements (SREs) that are necessary for transcriptional activity by free cholesterol, through the transcription factor SREBP-1 (Bist, Fielding, & Fielding, 1997). This transcriptional relationship may have evolved due to the observation by Smart *et al* that newly synthesized cholesterol utilizes cav-1 and caveolae to be

transported to the cell membrane (Mineo, James, Smart, & Anderson, 1996).

In terms of cholesterol homeostasis, caveolae have also been implicated in reveres cholesterol transport, the process during which excess free cholesterol is released into plasma via uptake by HDL particles. Evidence supporting this relationship includes: i) excess cholesterol appears in caveolae when cells are loaded with cholesterol; ii) anti-sense cav-1 reduces cholesterol efflux; and iii) adenoviral delivery of cav-1 to the liver results in increased plasma HDL cholesterol levels (Razani, Woodman, & Lisanti, 2002). in direct corroboration of these findings, SR-BI, a major receptor involved in cholesterol efflux to HDL, is significantly enriched within caveolae (Babitt et al., 1997). Finally, caveolae also appear to be involved in the uptake of cholesterol esters from the plasma (Fielding & Fielding, 2001).

3.2.7 Signal transduction

The establishment of a technique to isolate caveolae by virtue of their unique biochemical properties enabled large-scale

identification of other proteins residing in these membrane structures (Sargiacomo, Sudol, Tang, & Lisanti, 1993). Caveolae have now been demonstrated to concentrate a wide variety of proteins and signaling molecules including GPI-linked proteins, Src-family tyrosine kinase, H-Ras, hetereotrimeric G-proteins alpha subunit, PKC isoforms, and eNOS (for the complete list see (Razani, Woodman, & Lisanti, 2002)). Interestingly, a large number of these molecules have been demonstrated to interact directly with cav-1, many through a defined motif that has come to be termed the caveolin scaffolding domain (CSD, residues 82-101) (Couet, Li, Okamoto, Ikezu, & Lisanti, 1997). This led to the establishment of the “caveolin signaling hypothesis”, stating that caveolae/caveolin-1 do not solely function to compartmentalize and concentrate other proteins but also have a functional role in modulating signal transduction (Lisanti, Scherer, Tang, & Sargiacomo, 1994).

Direct proof of this notion was provided in several landmark experiments demonstrating that cav-1 directly inhibited activation of proto-typical cavolar residents, hetero-trimeric G proteins (S. Li et al., 1995). Since then, cav-1 has been shown to negatively regulate signaling of many other proteins, including eNOS (Garcia-Cardena

et al., 1997), EGFR (Couet, Sargiacomo, & Lisanti, 1997), Src tyrosine kinases (S. Li, Couet, & Lisanti, 1996), H-Ras (Engelman et al., 1997), and Neu (Engelman, Lee et al., 1998). These negative regulatory relationships are also preserved *in vivo*: cav-1 (-/-) deficient mice demonstrate the physiological hyper-activation of eNOS within their vasculature (Razani et al., 2001). Undoubtedly, the ability of cav-1 and caveolae to modulate signaling has important implications for signaling in transformed, neoplastic cells. The data for cav-2 as a signaling modulator is less clearly defined, perhaps due partly to more divergent CSD sequence. However, the cav-3 CSD maintains considerable homology to cav-1, and cav-3generated caveolae have been shown to compartmentalize and modulate a number fo signaling proteins, including eNOS, β-adrenergic receptors, PKC isoforms, G-proteins, Src-family kinases, and multiple components of the dystrophin-glycoprotein complexes (α-sarcoglycan, β-dystroglycan, and dystrophin) (Razani, Woodman, & Lisanti, 2002).

3.2.8 Cellular proliferation

The observation that cav-1 is expressed at the highest levels in terminally differentiated, relatively quiescent cells such as adipocytes, endothelium, smooth muscle cells and Type I penumocytes first a role for cav-1 in cell proliferation. Several groups have now demonstrated a role for cav-1 in cell cycle progression. Lee *et al* have documented that cav-1 levels are regulated during the cell cycle (S. W. Lee, Reimer, Oh, Campbell, & Schnitzer, 1998). In *C. elegans,* a role for cav-1 in meiotic cell cycle progression has been established (Scheel, Srinivasan, Honnert, Henske, & Kurzchalia, 1999). Galbiati *et al* have shown that cav-1 levels are regulated by growth factor stimuli and that overexpression can inhibit proliferation in mediating cell cycle arrest in G0/G1 (Galbiati, Volonte et al., 2001). Additionally, our laboratory has reported that in mouse embryonic fibroblasts (MEFs), genetic ablation of cav-1 results in increased proliferation rates concomitant with increased S-phase and decreased G0/G1 fraction, as well as altered expression of several cell cycle-related proteins (Razani et al., 2001; Williams et al., 2004).

Elucidation of the mechanisms by which cav-1 exerts negative regulatory effect on proliferation has uncovered a few of the molecular pathways involved. Cav-1 demonstrated to cause transcriptional suppression of Cyclin D1 an important regulatory component of the cyclin-cdk complex that phosphorylates Rb, thereby controlling entry into the S phase (Hulit et al., 2000). Furthermore, we have demonstrated that the induction of G0/G1 arrest by cav-1 overexpression appears to operate through a p53/p21^{Cip1} dependent pathway (Galbiati, Volonte et al., 2001).

The large majority of research on cav-1 and proliferation has centered on the ERK-1/2 (p42/44 MAP kinas) mitogenic signaling cascade. Many components of the ERK signaling cascade appear to be localized in caveolae, including growth factor receptors such a EGFR, TGF-βR, Ras, Ras-1, MEK, and ERK (Liu, Ying, & Anderson, 1997; Liu, Ying, Ko, & Anderson, 1996; Mineo, James, Smart, & Anderson, 1996; Rybin, Xu, & Steinberg, 1999; Smart, Ying, Mineo, & Anderson, 1995; Song, Li et al., 1996). Additionally, recombinant cav-1 inhibits activation and signaling ERK 1/2 to the nucleus both *in vitro* and *in vivo* (Engelman, Chu et al., 1998). In cells, down-regulation of cav-1 or genetic ablation of cav-1 has

been shown to be sufficient to result in constitutive hyperactivation of ERK 1/2 (Galbiati et al., 1998). Furthermore, hyperactivated ERK 1/2 has also been detected *in vivo* in cav-1 (-/-) mice under various condition (Capozza et al., 2003; Cohen et al., 2003). Interestingly, there appears to be a reciprocal relationship between ERK and cav-1, since activation of the ERK 1/2 cascade down-regulates cav-1 expression (Engelman, Zhang, Razani, Pestell, & Lisanti, 1999). Taken together, it appears that one of the function of cav-1 is to negatively regulate activation of the ERK 1/2 cascade.

3.2.9 Caveolin-1 and Apoptosis

Cell, including tumor cells, constantly face the decision of whether to survive and proliferate or commit to apoptosis, or programmed cell death. Therefore, identifying the signals or pathways inside a cell that are pro-apoptotic or anti-apoptotic has important implications for controlling tumor cell growth. Unfortunately, the evidence of the role of cav-1 in apoptosis is somehow contradictory and disparate. On the one hand, caveolae are highly enriched in both sphingomyelinase, the enzyme responsible for generating ceramide (Liu & Anderson, 1995).

Ceramide induces cell death by inhibiting the activation if the well-characterized anti-apoptotic/survival pathway phosphatidylinositol 3-kinase (PI3-K)/AKT (Zundel & Giaccia, 1998). Interestingly, cav-1 has been shown to interact with the PI3K and cav-1 overexpression sensitizes fibroblasts to ceramide-induced death through a PI3-K-dependent mechanism (Zundel, Swiersz, & Giaccia, 2000). Furthermore, Cai *et al* mentioned an alternative pathway that cav-1 can sensitize cancer cells to drugs by inhibition of P-glycoprotein, which is an drug efflux transporter (Cai & Chen, 2004).

On the other hand, negative regulation of cav-1 activity through distribution of caveolae by cholesterol-sequestering agents has been shown to block IL-6 and IGF-1induced activation of the PI3-K/AKT signaling pathway (Podar et al., 2003). Therefore, caveolae and cav-1 are required to mediate proper survival signals through the PI3-K/AKT pathway. Moreover, cav-1 overexpression in Rat1 cells and human prostate cancer cells (LNCaP), or cav-1 up-regulation I androgen-insensitive LNCaP clones renders these cells more resistant to apoptosis (Timme et al., 2000; Tso et al., 2000). In addition, anti-sense down-regulation of cav-1 results in prostate cancer cells that are more sensitive to apoptosis (Tso et al., 2000).

Finally, Li *et al* have demonstrated that cav-1 overexpression mediates cell survival by sustaining activation of Akt through binding and inhibition of the serine/theronine protein phosphatases PP1 and PP2A (L. Li, Ren, Tahir, Ren, & Thompson, 2003). Taken together, these results argue that cav-1 has anti-apoptotic activities.

The apparent incongruity of pro-apoptotic or anti-apoptotic function of cav-1 can be explained by cell-type specific roles for cav-1 (i.e. anti-apoptotic in prostate cancer cells) or may be due to the use of different inducers of apoptosis. In summary, these finding define multiple cell-type and apoptotic pathway-specific roles for cav-1 in cell survival and death.

3.2.10 Caveolin-1 and transformation

An inverse relationship between cav-1 expression and transformation has now been clearly established. During the initial characterization of cav-1, it was demonstrated that cav-1 levels were reduced in transformed cells and that the level of cav-1 inversely correlated with soft agar growth (Koleske, Baltimore, & Lisanti, 1995). Subsequently, multiple groups demonstrated that

forced re-expression of cav-1 could significantly attenuate soft agar (anchorage independent) growth in transformed cells (Engelman et al., 1997; S. W. Lee, Reimer, Oh, Campbell, & Schnitzer, 1998; Razani et al., 2000). Interestingly, reductions of cav-1 by an anti-sense strategy were sufficient to induce a transformed phenotype in NIH-3T3 cells, allowing these cells to grow in soft agar and form tumors in immunodeficient mice (Galbiati et al., 1998). Furthermore, another group has recently demonstrated that cav-1 haploinsufficiency achieved through a retrovirus-mediated gene trapping approach is sufficient to induce partial transformation in human breast epithelial cells (Zou, McDaneld, & Smith, 2003).

The utilization of cav-1 null mice has provide molecular genetic evidence contributing to the notion that cav-1 serves as an anti-transformative agent in many tissue. Cappozza *et al* show that the skin of cav-1 (-/-) mice is more susceptible to chemical carcinogenic treatment, resulting in the formation of benign epidermally-derived tumors (Capozza et al., 2003). In addition, Williams *et al* demonstrated that the loss of cav-1 accelerates the appearance for mammary dysplastic lesions in tumor prone transgenic mice (Williams et al., 2003). Finally, genetic ablation of

cav-1 in MEFs renders them more susceptible to transformation and *in vivo* tumorigenesis with transforming oncogenes (Williams et al., 2004). Through the use of mice lacking one or both alleles of cav-1, these reports mimic the effects of naturally occurring inactivating genetic mutations.

3.2.11 Tumor suppressor and oncogenes

Research on the relative relationship of cav-1 to other cellular oncogenes and tumor suppressors bears importance for the intrinsic working of the cells. The multi-step nature of cellular transformation involves the acquisition of genetic alteration that ultimately affect the transcription, translation, or post-translational regulation of proteins, the functional end-product of DNA. These mutations can be transmitted through the germline, or can be acquired somatically during the lifetime of a cell. Two important properties of neoplastic cells are immortalization and transformation. In some sense, all cells within a microenvironment are undergoing the process of natural selection, and the cells that are able to more quickly attain those two properties will more likely survive and propagate. Therefore, it is important to determine the

relative contributions of different genes in promoting immortalization or transformation.

While cav-1 does not appear to have a direct role in immortalization, cav-1 does synergize with other immortalizing genes. Loss of the INK4 locus, which lost in 95% of pancreatic tumors –as we previously explained- (Caldas et al., 1994; Schutte et al., 1997), encoding both $p16^{INK4a}$ and $p19^{ARF}$, is sufficient for certain cells to become immortalized. Williams *et al* demonstrated that concomitant loss of cav-1 with INK4a, results in cells with a proliferative advantage, demonstrating that the loss of cav-1 expression cooperates or synergizes with the genetic mutations that abolish INK4a function (Williams et al., 2004). Furthermore, transformation of immortalized cells INK4a (-/-)/cav-1 (-/-) with various oncogenes renders these cells more neoplastic, with large *in vivo* tumor burden. This data suggests that mutations or down-regulation of cav-1 expression in certain cell types, in combination with an INK4a mutation, would impart cells with a profound neoplastic advantage over those cells with a mutation in either gene alone.

Another well-characterized tumor suppressor, p53, appears to be directly involved in regulating cav-1 expression. Razani *et al* have demonstrated that p53, which is inactive in 75% of pancreatic cancer, is a positive transcriptional and translational regulator of cav-1, and that inactivation of p53 through viral oncoproteins results in reduced cav-1 expression (Razani et al., 2000). cav-1 levels are also dramatically reduced in p53-deficient cells (S. W. Lee, Reimer, Oh, Campbell, & Schnitzer, 1998).

A whole host of cellular oncogenes have been shown to reduce cav-1 expression in cells (Tabel.4). These include, but are not limited to, c-Myc, HPV E6, v-abl, bcr-abl, H-Ras^{G12V}, v-Src, Neu/ErbB2 (Engelman, Lee et al., 1998; Koleske, Baltimore, & Lisanti, 1995; Park et al., 2001; Razani et al., 2000; Timme et al., 2000). Virtually all of these oncoproteins appear to down-regulate cav-1 expression through a transcriptional mechanism. Additionally, Ras and Raf-mediated downregulation of cav-1 relies upon ERK 1/2 activation as ERK 1/2 inhibition restores cav-1 expression in Ras and Raf-mediated cells as well as in human fibrosarcoma cells (Engelman, Zhang, Razani, Pestell, & Lisanti, 1999; Wiechen et al., 2001). Activation of non-oncogenic proteins have also been

demonstrated to down-regulated cav-1 expression, including PKA and PKC alpha (Engelman, Zhang, Razani, Pestell, & Lisanti, 1999; Xie, Zeng, Waldman, & Glazer, 2003).

3.2.12 Cavolin-1 in human cancers

The human chromosomal location of the cav-1 gene has been mapped closely to the D7S522 microsatellite repeat marker locus on 7q31.1 (Engelman, Zhang, & Lisanti, 1998). The observation that this region is commonly deleted in a number of human cancers, including carcinomas of the pancreas, breast, colon, kidney, prostate, ovary, head and neck has led to hypothesis that this region encodes a tumor suppressor locus (Achille et al., 1996; Matsuura et al., 1998; Zenklusen, Bieche, Lidereau, & Conti, 1994; Zenklusen, Thompson, Klein-Szanto, & Conti, 1995; Zenklusen, Thompson, Troncoso, Kagan, & Conti, 1994; Zenklusen, Weitzel, Ball, & Conti, 1995).

However, sequence analysis of cav-1 in human tumors has so far revealed only a few mutations. As previously mentioned, Hayashi *et al.* detected a sporadic P132L mutation in up to 16% of

patients with primary breast cancer (Hayashi et al., 2001). Molecularly, this mutation induces cellular transformation, acts in a dominant negative fashion by causing mislocalization and intracellular retention of wild-type cav-1, and causes ERK activation (Hayashi et al., 2001; H. Lee et al., 2002). Interestingly, this mutation appears in he analogous residue (P104L) in cav-3 in patients with an autosomal dominant form of limb-girdle muscular dystrophy (Minetti et al., 1998). Independently, another group has identified novel cav-1 mutations in human oral squamous cell cancer (S. E. Han, Park, Lee, Huh, & Min, 2004). These mutations await further molecular characterization.

Cav-1 has now been implicated in a wide range of human tumors. Interpretation of the cellular data on inhibition of transformation by cav-1 proposes that the majority of human tumors would demonstrate reduced levels of cav-1. This is clearly true for other proteins, such as the well characterized p53 or Rb tumor suppressors, where a consistent pattern on down-regulation or inactivating mutation of one or both alleles is observed across a large variety of tumor types. On the contrary, cav-1 expression level is not consistently down-regulated in the majority of human tumor

types. However, within tumor types derived from the same cell type or tissue, cav-1 expression levels are consistently wither up-regulated or down-regulated in the majority of cases. For instance, cav-1 down-regulation is typical of ovarian, lung and mammary carcinomas, as well as mesenchymal sarcomas. On the other hand, cav-1 is consistently up-regulated in bladder, esophagus, thyroid, and prostate carcinomas. As there are always exceptions to the rule, cav-1 expression has been detected in intraductal and infiltrating breast carcinomas, while some androgen-insensitive prostate cancer cells demonstrate reduced cav-1 levels (Pflug, Reiter, & Nelson, 1999; Yang et al., 1998). Variable expression patters have been observed in colon, renal, oral and pancreatic carcinomas. Further research will assist in identifying the modifying factors that determine whether it is the up-regulation or down-regulation of cav-1 in these latter tumor types that facilitates tumorigenesis.

3.2.13 Caveolin-1 and pancreatic cancer

Although little is known about cav-1 in pancreatic cancer. cav-1 data shows contradiction between *in vivo* and *in vitro.* Although Suzuoki *et al* found that cav-1 is a negative predictor of survival as it positively correlates with tumor diameter, histopathologic grade and poor prognosis (Suzuoki et al., 2002), on the other hand, several *in vitro* studies described cav-1 role as a tumor suppressor molecule. Indeed, Lin *et al* showed that cav-1 inhibits migration and invasion ability of pancreatic cancer cell lines (Lin, DiVito, Merajver, Boyanapalli, & van Golen, 2005), And Han *et al* mentioned that cav-1 decreases invasion through inhibition of matrix metalloproteinases (MMPs) (F. Han & Zhu, 2009). Moreover, Han *et al* showed that cav-1 decreases tumor growth in nude mice (F. Han, Gu, Chen, & Zhu, 2009). This contradiction of data encouraged us to investigate and verify the cav-1 role through several *in vitro* and *in vivo* studies.

3.3 Epithelial-mesenchymal transition

3.3.1 Introduction

Epithelial-mesenchymal transition (EMT) is a biological process where it allows polarized arranged epithelial cells, which are connected to a basement membrane on the basal side, to undergo through biochemical and molecular alteration to gain a mesenchymal cell phenotype. EMT was first described by Elizabeth Hay when she noticed transformation of epithelial cells to mesenchymal and vice versa using chick primitive streak formation model (Hay, 1995). While epithelial can change to mesenchymal and vice versa due to cell plasticity, the word transformation has changed to transition. EMT is initiated by variant molecular processes such as various transcriptional factors, expression or loss of membranous proteins, reorganization if cytoskeletal proteins and production of extracellular matrix enzymes. Kalluri *et al* proposed three different biological subtypes of EMT, based in the biological cell context (Kalluri & Weinberg, 2009). Type 1 EMT is associated with implantation, embryo formation, and organs development, in this type epithelial cells transit to mesenchymal and vice versa -mesenchymal-epithelial transition- (MET); type 2 EMT is associated with inflammation, fibrosis and wound healing;

type 3 EMT occurs in neoplastic cells, which is characterized by high migratory and invasiveness potential, elevated apoptosis and drug resistance and increased production of extracellular matrix (ECM) (Figure.7) (Kalluri & Weinberg, 2009). Various molecular alterations are accompanied with EMT process which allow cells to lose epithelial cell-cell junction, reorganization of actin cytoskeleton, loss of epithelial proteins that promote cell-cell contact such as E-cadherin, β- and γ-catenin, and zonula occludens-1 (ZO-1), and gain of mesenchymal markers such as vimentin, α-smooth muscle actin, N-cadherin, Snail and Slug.

Loss of E-cadherin is considered the hallmark of EMT. E-cadherin is a membranous adhesion molecule and a calcium-dependent glycoprotein, which is responsible for cell-cell contact and cell tight junctions in epithelial cells (Makrilia, Kollias, Manolopoulos, & Syrigos, 2009). E-cadherin cytoplasmic portion forms a complex with β-catenin. This complex stabilizes β-catenin on the plasma membrane and prevents β-catenin translocation to the cytosol. Cytosolic β-catenin is ubiquitinated by the degradation complex that consists of serine/threonine kinases CK1, glycogen synthase kinase-3β (GSK-3β), the adenomatous polyposis coli

protein (APC), and the scaffold protein axin, or β-catenin is translocated to the nucleus when the canonical Wnt pathway is active (Schmalhofer, Brabletz, & Brabletz, 2009). Nuclear β-catenin binds with transcriptional complex T-cell factor (TCF)/lymphoid enhancer factor (LEF) which targets many oncogenes (Q. Wang, Sun, Allgayer, & Yang, 2010). This illustrate the importance of E-cadherin role for maintaining cell phenotype and normal behavior. Many external inhibitors of E-cadherin and initiators EMT were identified such as transforming growth factor-β (TGF-β), fibronectin, epidermal growth factor (EGF) and platelet-derived growth factor (PDGF) (Camara & Jarai, 2010; M. Y. Lee, Chou, Tang, & Shen, 2008; Sabbah et al., 2008). These external molecules initiates many signal transduction pathways such as Phosphoinositide 3-kinases (PI3K)/ AKT, Mitogen-activated protein kinases (MAPK)/ Extracellular Receptor Kinase (ERK) and Smads (Camara & Jarai, 2010; Sabbah et al., 2008). Activations of these signal pathways increase transcriptional inhibitors of E-cadherin such as zing finger binding transcriptional factors, namely Snail and Slug or as basic helix-loop-helix transcriptional factors, namely twist, Zinc finger E-box-binding homeobox (ZEB) -1 and -2 (von Burstin et al., 2009; Voulgari & Pintzas, 2009). These transcriptional factors inhibit E-cadherin through binding to the E-cadherin promoter site (E-box).

3.3.2 EMT in drug resistance and metastasis

Several recent studies have demonstrated that EMT is associated with drug resistance and metastasis (Cheng et al., 2007; Christiansen & Rajasekaran, 2006; DiMeo et al., 2009; Fuchs et al., 2008; Kudo-Saito, Shirako, Takeuchi, & Kawakami, 2009; Sarkar, Li, Wang, & Kong, 2009; Tsuji, Ibaragi, & Hu, 2009; Voulgari & Pintzas, 2009). Also, many emerging evidences have indicated EMT role in tumor progression (Thiery, 2002). EMT acquires cancer cells with mesenchymal phenotype which is characterized by loose embedding and loss of polarity (Kalluri & Weinberg, 2009). This phenotype gives cancer cells infiltrating and metastasis potential. Recently, Mendez *et al* found that metastatic cells express the mesenchymal marker, vimentin, and demonstrate spindle-shape morphology and more motility ability (Mendez, Kojima, & Goldman). Moreover, Arumugam *et al* showed that pancreatic cell lines that showed low expression of E-cadherin and high expression of mesenchymal marker, ZEB1, demonstrated high resistance to three conventional drugs used in pancreatic cancer treatment, gemcitabine, 5-FU, and cisplatin (Arumugam et al., 2009). Furthermore, Yin *et al* showed that Snail overexpression in

pancreatic cancer cells enhanced metastasis and chemoresistance (Yin et al., 2007).

Here in this study, we propose cav-1 as a crucial modulator of EMT and cell differentiation. We detected cav-1 at pretranslation and translational levels in eight pancreatic cell lines. We chose the least cav-1 expressing –Panc 10.05- (Panc10/cav-1) cell line to infect it with cav-1 expressing vector. Consequently, cancer cells acquired different morphological phenotype and more cell adherens than control cells. After that, levels of E-cadherin and β-catenin were higher and localized in cell membrane. This was combined with diminishing of the E-cadherin inhibitor, Snail. Also, molecules such ERK, Smad2 were dephosphorylated and AKT was downregulated in cav-1 expressing cells. Prior cellular and molecular alternations decreased cancer cells migration and invasion potential, plus sensitized cancer cells toward doxorubicin. Also, we grown cells overexpressing cav-1 in three-dimensional culture to investigate cell polarity and behavior. Also, we studied the cell cycle of a metastatic cell line incubated with cav-1 peptide. Furthermore, *in vivo* study in nude mice showed that cav-1 expressing tumors were less in weight and volume compared to

control tumors. Interestingly, cav-1 tumors contained nests of differentiated cells nests which were absent in control tumors. These differentiated cells exhibited strong E-cadherin and β-catenin signals in cell membrane while Snail was absent in cells cytoplasm or nucleus.

4. Material & Methods

4.1 Cell culture, stable retroviral transfection.

Panc 10.05, Mia Paca, BxPC3, Aspc-1 and human pancreatic duct epithelial (HPDE) cell lines were purchased from American Type Culture Collection. HS766T and PK9 cell lines were a gift from Scott Kern (John Hopkins University), and HPAF II cell line was a gift from Surinder Batra (University of Nebraska Medical Center). All cell lines were maintained at 37 °C in 5% CO_2 and grown in RPMI 1640 (Gibco; Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS) (Gibco), except Panc 10.05, where 10 I.U./ml of human recombinant insulin (Sigma-Aldrich Co.; St Louis, MO, USA) were added to the growing medium. Full-length cav-1 gene was subcloned in the pBabe retroviral vector using standard PCR (Capozza et al., 2005). Then, Phoenix, amphotropic packing cell line, was trasfected with pBabe vectors using a modified calcium phosphate method (Kinsella & Nolan, 1996). After transfection for 48 hours, viral supernatant was collected, filtered, and added to Panc 10.05 cells. Two cycles of infection were added every 12 hours. Puromycin (Sigma-Aldrich Co.) was added after the final infection cycle at a final concentration of 2.5μg.ml^{-1}. Finally, the success of cav-1 infection was confirmed by Western blot analysis.

4.2 Real-time PCR analysis

Cells were grown in 10 cm Petri dish (Corning Glass Works, Corning, NY) till 80% confluence. Then, culture medium was aspirated and cells were rinsed twice with PBS (Gibco). 1 ml of Trizol (Sigma-Aldrich Co.) was added to the plates and swirled. After that, Trizol suspension was transferred to tubes contain 200 µl chloroform (Sigma-Aldrich Co.). The tubes were vortexed, then centrifuged for 10 minutes at 13000 RPM. The clear upper zone, which has the RNA, was transferred to tubes contain 400 µl isopropanol (Sigma-Aldrich Co.) and vortexed. The mixture was left to settle for overnight at room temperature (RT). Next day, the tubes were centrifuged for 20 minutes at 13000 RPM, the supernatant was carefully aspirated and discard. Washing of RNA pellet was achieved by adding 100 µl of 75% Ethanol, and the mixture was centrifuged. Then the Ethanol was aspirated, and the pellet were left to dry for 30 minutes in RT. To dissolve the pellet, we added 100 µl of DEPC-treated water (Ambion; Austin, TX, USA) and left to dissolve for 30 minutes at 65°C. RNA quality and quantity was determined by using The NanoDrop ND-1000A Spectrophotometer (Ambion). Then, cDNA was synthesized from the purified mRNA using SuperScript III First-Strand (Invitrogen;

Carlsbad, CA, USA) according to the manufacture's instruction. Cav-1 and glyceraldehyde-3 phosphate dehydrogenase (GAPDH) probes were purchased as "assays on demand" (Applied Biosystems; Foster City, CA, USA) and GAPDH was used as housekeeping gene. cDNA was prepared, and subjected to real-time PCR using the TaqMan technology (7500 Sequence Detector; Applied Biosystems). Detection was carried out in duplicates.

4.3 Immunoblotting assays

Cells were lysed using RIPA buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 0.5% Sodium Deoxycholate, 1.0% Triton X-100, 0.1% SDS), plus protease inhibitors (Roche Diagnostics; Indianapolis, IN, USA) and phosphatase inhibitors (Sigma-Aldrich Co.). Cell lysates were centrifuged to remove cell debris. Protein quantification was achieved by using BSA reagent (Pierce; Rockford, IL, USA) and the desired volume, which contains 50 μg protein, was determined. Cell lysate proteins were separated by 10-12% SDS-PAGE electrophoresis then transferred overnight at 4°C to nitrocellulose membrane (Whatman; Dassel, Germany). The antibodies used for immunoblotting were the rabbit polyclonal anti-caveolin-1 (N-20), and the mouse monoclonal anti-GAPDH were

purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA), the mouse monoclonal anti-E-cadherin, the mouse monoclonal anti-β-catenin, and the mouse monoclonal anti-Akt were purchased from BD Biosciences (San Jose, CA, USA), the rabbit polyclonal anti-Snail, the rabbit polyclonal anti-Smad2, the rabbit polyclonal phospho-Smad2 (465/7), the rabbit polyclonal anti-Erk1/2, the rabbit polyclonal anti-phospho-Erk1/2 (202/4), and the rabbit polyclonal anti-phospho-Akt (Ser473) were purchased from Cell Signaling Technologies (Danvers, MA, USA). Some of the later antibodies were used for immunofluorescence and immunohistochemistry.

4.4 Immunofluorescence assay and confocal microscopy

Panc10/cav-1 cells were plated on cover slips in 12-well plates and cultured for two days. Then cells were rinsed with 0.1 mM $CaCl_2$ and 1mM $MgCl_2$ in PBS (PBS/CM), then fixed with 2% paraformaldehyde in PBS/CM for 30 minutes. After, cells were washed three times with PBS/CM , and then permeabilized with 0.1% Triton-X100 and 0.2% bovine albumin serum in PBS/CM (IF buffer). After cells were quenched with 50 mM NH_4Cl in PBS/CM for

10 minutes, cells were rinsed with IF buffer. Cells were incubated with (1:500) and E-cadherin (Mouse) (1:200), or β-catenin (Mouse) (1:200) antibodies for 1 hour at room temperature. Then cells were washed with IF buffer and incubated with secondary antibody, Alexa Fluor goat anti-mouse 488 IgG (Molecular Probes; Eugene, OR, USA) for 30 minutes. After washing cells with IF buffer, cells were rinsed with PBS alone. Nuclei were stained with Hoechst 33342 for 2 minutes, then cells were rinsed with PBS. Coverslips were mounted with ProLong Gold anti-fade (Molecular Probes) and left overnight in the dark. Images were acquired with a Zeiss LSM510 Meta confocal microscope system and analyzed with Zeiss LSM Browser (3,5,0,359; Zeiss).

4.5 Akt activity

Akt kinase activity was measured using nonradioactive AKT Kinase Assay kit (Cell Signaling Technologies). According to the manufacture's instruction, stable infected Panc 10.05 cell lysates were incubated with immobilized Akt antibody beads for overnight at 4°C. Next day, the solution was centrifuged and the pellet was washed twice. Then, the pellet was suspended and incubated with the glycogen synthase kinase-3 α/β (GSK-3α/β) fusion protein for

30 minutes at 30 °C. After the reaction was stopped with 20 µl of 3X SDS, the samples were transferred to be immuneblotted with phospho-GSK-3 α/β (Ser21/9) antibody (Cell Signaling Technologies).

4.6 Migration and invasion assays

Cells invasion potential *in vitro* was measured using modified Boyden chamber assay (Albini et al., 1987; Hulit et al., 2007). Briefly, 2.5 x 10^4 Panc10/cav-1 and Panc10/pBabe cells were suspended in 0.5 ml of serum-free RPMI-1640, then added to 8 µm-pore upper chamber (BD Biosciences). The upper chambers were either coated with Matrigel, for invasion assay, or not coated, for migration assays. The lower chambers contained RPMI-1640 medium supplemented with 10% FBS served as a chemoattractant. After that, cells were incubated at 37 °C for 10 hours or 20 hours to migrate or invade, respectively. The non-invasive cells on the top of the upper chamber were removed using a cotton swap, then the invasive cells were stained with 0.5% crystal violet dissolved in methanol for 30-60 minutes. Chambers were rinsed with water, dried, and then examined under bright-field microscope. Invasion and migration values were obtained through counting 5 fields of

each chamber. The assay were carried out in triplicates in different time points.

4.7 Drug sensitivity assay.

Panc10/cav-1 were plated in 96-well dish. After 24 hours, cells medium was discarded, then a serial dilution of doxorubicin hydrochloride (Sigma-Aldrich Co.) were added to the cells for 48 hours incubation. Cells survival was detected using CellTiter 96 non-Radioactive Cell proliferation Assay kit (Promega; Madison, WI, USA). Survived cells formed a color solution which was detected at 570nm wavelength according to the manufacture's instruction using an ELISA reader (Sun-rise Remote, TECAN; Austria) . Each concentration was performed in triplicates.

4.8 Three Dimensional Cultures

Matrigel of basement membranes extract of reduced growth factors (Cultrex; Gaithersburg, MD, USA) was defrosted and 100 µl of the Matrigel was transferred to cooled chamber slides (BD Falcon; Bedford, MA, USA). Then matrigel was evenly spreaded

with a 20 µl cooled tip. Slides were incubated in 37 °C for maximum of 3 hours to solidify. Cells were washed with cooled PBS twice, then they were trypsinized. Then cell suspension was collected with growth media and spinned for 4 minutes at 1000 RPM. Then the cells pellet was resuspended until the pellet was dispersed to single cell suspension. Cells were counted and then they were diluted to a final concentration 5000 cells/ml. The same volume of the cell suspension was mixed with equal volume of growth medium containing 4% of matrigel reduced growth factors plus 10 ng/ml of EGF (BD Bioscience) to reach a final cell suspension of 10000 cells with a 2% Matrigel plus 5 ng/ml EGF. The later suspension was transferred to chamber slide well that contains the solidified matrigel. Slides were incubated at 37 °C in 5% CO_2 for 3 days. Images were acquired by an Axiovert 200 M microscope (Carl Zeiss; Oberkochen, Germany). This experiment was carried out in quadruplicates.

4.9 Caveolin-1 peptide and cell cycle

The scaffolding domain of Caveolin-1 was coupled with a 16-amino acid peptide which is corresponding to the homeodomain of the *Drosophila* transcription factor *antennapedia* (AP), which

facilitate the uptake of AP-caveolin-1 (AP-cav-1) into mammalian cells through a nonendocytosis and nondegrative pathway (Derossi et al., 1996; Gratton et al., 2003). Biotinylated peptides corresponding to AP [(biotin)-*RQPKIWFPNRRKPWKK*-(OH)] and AP-cav-1[(biotin)-*RQPKIWFPNRRKPWKK*–DGIWKASFTTFTVTKYWFYR-(OH)] were synthesized (at the Tufts University Core Facility). 5mM of the peptide, dissolved in the growth media, was incubated with Aspc-1 cell line in 37 °C for 48 hours. Cells were washed by PBS, then trypsinized. Trypsinized cells were collected then spinned for 8 minutes at 1200 RPM. Supernatant was discarded and the pellet was washed by cold PBS twice. Then, cells were dispersed in 70% ethanol to fix the cells overnight on the shaker at 4 °C. Next day, cell suspension was spinned, and ethanol supernatant was discarded. Cell pellet was washed with cold PBS once, then spinned. Cell pellet was suspended in 500 µl with the staining solution containing 0.05mg/ml propidum iodide (Invitrogen) plus 5 µg of RNAse A (Roche Applied Science; Indianapolis, IN, USA) for 30 minutes in 37 °C water bath in dark. Then, cell suspension was transferred to cell sorting tube. Cell cycle was determined by flow cytometry (Coulter Epics XL MCL software; Coulter Epics XL MCL System II 3.0; Beckman Coulter, Miami, FL).

4.10 Animal study

All nude mice in this study were conducted according to the guidelines of the National Institutes of Health and the Thomas Jefferson University Institute for Animal Studies. Male nude mice at the age of 8 weeks were obtained from NIH. The mice were divided to two groups, each group had nine mice, then every mouse was injected in the left flank with 1 x 10^6 cells. After 7 weeks tumors appeared, extracted, then weighted and measured. Finally, tumors were embedded in paraffin blocks for further immunohistochemistry analysis.

4.11 Immunohistochemistry

Tumor slides were processed as we described before (Bonuccelli et al., 2009). Briefly, tumors embedded in paraffin were cut in 5 μm sections. Then, sections were deparaffinized with xylene and rehydrated with graded concentration of ethanol. Antigen retrival was performed in 100 mm/L sodium citrate buffer pH 6.0 for 10 minutes using an electric pressure cooker. Then sections were incubated with 3% H_2O_2 for 10 minutes, then sections

were washed and blocked with 10% goat serum in PBS for 1 hour at room temperature (RT). After that, slides were washed and incubated with primary antibodies anti-E-cadherin (1:400) or anti-β-catenin (1:200) or anti-Snail (1:250) in blocking solution for overnight at 4 °C followed by incubation with biotinylated secondary antibody for 30 minutes at RT. Then, slides were washed in PBS and incubated with avidin/biotin-horesradish peroxidase reagent for 30 minutes at RT. Next, slides were washed again and incubated with the 3,3-diaminobenzidine reagent until developing a brown color. Finally, slides were washed, then counterstained with hematoxylin, dehydrated, then mounted with coverslips.

4.12 Statistical analysis

Statistical significance was examined by Student's *t*-test. Values of $P < 0.05$ were considered significant or lower were highly statistically significant. Values were expressed as means ± SE (Standard Error).

5. Results

5.1 Differential expression of caveolin-1

To determine the lowest expressing pancreatic cell line to cav-1, we performed mRNA relative quantification and Western blot studies (Figure.8A and 8B). Immunoblotting study demonstrated that Panc 10.05 cell line has the lowest amount of cav-1 (Figure.8B). So, we used Panc 10.05 cell to establish a stable infection of cav-1 expressing Panc 10.05 (Panc10/cav-1) (Figure.8C).

5.2 Cells expressing cav-1 acquire polygonal morphology, have more cell-cell contact and express E-cadherin and β-catenin in the plasma membrane

Subsequently to the cav-1 infection, Panc10/cav-1 exhibited different morphology than Panc 10.05 cells infected with empty vector (Panc10/pBabe). Panc10/cav-1 exhibited more cell-cell contacts and polygonal shape, in the contrary Panc10/pBabe cells were growing solely and exhibiting a fibroblastic spindle-shape (Figure.9A). This alteration of cell-cell behavior in Panc10/cav-1 was accompanied with elevation of E-cadherin expression, which is shown in figure.9B. Furthermore, immunofluorescence analysis

displays membranous expression of E-cadherin in Panc10/cav-1 cells (Figure.9C). Consequently to E-cadherin expression, β-catenin expression was restored and was stabilized in the plasma membrane of Panc10/cav-1 cells (Figure.9B and 9C).

5.3 Cav-1 suppresses EMT pathways

To investigate the mechanism by which cav-1 influence in E-cadherin restoration and suppression of EMT, we performed immunoblotting analysis against several critical molecules that are responsible for E-cadherin suppression. Interestingly we found that, ERK and Smad2 activity were lower (Figure.10A), and total AKT and Snail expressions were down-regulated in cav-1 expressing cells compared to control lysates (Figure.10B). Previous studies highlighted ERK and Smad role in EMT induction (Davies et al., 2005). Additionally, AKT activity analysis demonstrates reduced phospho-GSK-3*α/β* level (Figure.10C), which was attributed to reduced AKT activity in cav-1 expressing cells. AKT and its pathway has an immense influence in EMT induction (Grille et al., 2003). Mainly this influence is through the expression of the transcriptional factor, Snail, which down-regulates E-cadherin to initiate EMT.

5.4 Cav-1 attenuates pancreatic cancer cell behavior such migration and invasion, and drug resistance

AKT and ERK molecules play important role in cancer cells invasion and migration. To investigate if cav-1 effect over these two molecules can alters cells migration and invasion capacity, we seeded Panc10/cav-1 cells over 8 μm-pore Transwells, which were not coated or not coated with Matrigel for migration and invasion, respectively. As we expected, cav-1 expression dramatically decreased migration and invasion capacity approximately by 2.5 and 16-folds, respectively, as compared to Panc10/pBabe capacity (Figure.11A). Han *et al* explained that cav-1 decreases invasion potential via inhibition of matrix metalloproteases (MMP)-2 and MMP-9 secretion and the reduced ERK activity is responsible for migration and invasion inhibition (F. Han & Zhu, 2009). Doxorubicin is a promising cytotoxic drug, especially when is combined with Akt inhibitors (Y. A. Wang, Johnson, Brown, & Dobson, 2009). As we have shown before that cav-1 inhibits AKT activity, so we have chosen doxorubicin to study cav-1 expressing cells chemosensitivity. A serial dilution of doxorubicin were added to Panc10/cav-1 cells. Panc10/cav-1 cells demonstrated significant sensitivity to doxorubicin than Panc10/pBabe cells did (Figure.11B).

We suggest that this sensitivity is due to inhibition of EMT initiators and pro-survival molecules such AKT and ERK by cav-1 expression.

5.5 Cav-1 inhibits cancer cells loss of polarity, acini irregularity and branching in three-dimensional culture

As we mentioned before, that EMT is a biological process which potentiates tumor progression by the loss of polarity (Moreno-Bueno, Portillo, & Cano, 2008). Also, cav-1 was found to be an important regulator of cell polarity (Grande-Garcia & del Pozo, 2008; Grande-Garcia et al., 2007). So to determine the cav-1 expression role in EMT and cell polarity, we used three-dimensional culture which is used for investigating of cell polarity behavior (Shaw, Wrobel, & Brugge, 2004). We overlaid cells over Matrigel reduced from growth factors, then cells were incubated with growth medium supplemented with 2% of Matrigel and stimulated with 5 ng/ml EGF. After 3 days cav-1 deficient (Panc10/pBabe) cells displayed branching (tubulogenesis) morphogenesis and more proliferation and irregularity (irregular outline, multilobular). Debnath *et al* described that branching in three-dimensional culture requires

EMT (Debnath & Brugge, 2005). While Panc10/cav-1 cells showed dramatic decrease of branching and irregular spheres, and significant increase of polarized regular spheres formation (Figure.12).

5.6 Cav-1 peptide induces G_0/G_1 arrest

Cav-1 scaffolding domain peptide coupled with *antennapedia* (AP-Cav-1) was found to be permeabilized into cell membrane and reduces inflammation, and tumor progression in mice (Bucci et al., 2000; Gratton et al., 2003). In this study we wanted to demonstrate the effect of AP-Cav-1 peptide on cancer cell cycle. Using flow cytometry, Aspc-1 cells that treated with AP-Cav-1 showed significant induction of G_0/G_1 arrest and significant reduction of S and G_2/M Phases (Tabel.5).

5.7 Cav-1 impairs tumorigenesis and shrinks tumor mass and volume

Panc 10.05 cell line is tumorigenic, to evaluate if cav-1 can impair tumorigenesis potential of Panc 10.05 cell line after cav-1 infection , we injected infected cells to mice flanks. After 7 weeks,

tumors were extracted and weighed and volumes were measured as an ellipsoid which $V = \frac{4}{3} \text{ X } \pi \text{ X } a \text{ X } b^2$, where V is the tumor volume, a is the length of the long axis, and b is the length of the short axis. Surprisingly, Panc10/cav-1 tumor mass and volume were decreased roughly 9 and 4-folds, respectively, compared to Panc10/pBabe tumors (Figure.13A).

5.8 Cav-1 expression restores cells differentiation

Astonishingly after H&E staining, Panc10/cav-1 tumor displayed multiple nests of organized cells layers displaying semi differentiation towards squamous architecture (Figure.13B). These cells were surrounded by eosinophilic fibroblasts, giving a structure similar to epithelial cells surrounded by a basement membrane. However these nests were found in 7 out of 9 cav-1 expressing tumors, they were totally absent in control Panc10/pBabe tumors . These nests confirm that cav-1 plays a critical role in restoring cells differentiation and also these differentiated cells recruited fibroblasts from the environment to act as a basement membrane.

5.9 E-cadherin and β-catenin are expressed and Snail production is lost in cav-1 tumor cell nests

To examine if the differentiated nests have different E-cadherin and β-catenin expression level than the undifferentiated cells, we performed immunohistochemistry analysis. Indeedly, differentiated cells showed high membranous signal for E-cadherin and β-catenin (Figure.14). Moreover, Snail signal was absent from nest cells cytoplasm and nucleus compared to the surrounding undifferentiated cells. All of the *in vivo* results are consistent with *in vitro* results, which explicit the critical role of cav-1 in suppression of EMT in pancreatic cancer.

6. Discussion & Conclusion

In this study we wanted to evaluate caveolin-1 (cav-1) regulatory role in the E-cadherin expression and epithelial to mesenchymal transition –EMT- in pancreatic cancer. Pancreatic cancer is considered to be the fourth leading cause of death related to cancer due to its drug resistance and rapid metastasis (Sarkar, Li, Wang, & Kong, 2009). These features have been related to EMT (Sarkar, Li, Wang, & Kong, 2009). EMT is a biological and molecular process in which epithelial phenotype and cell polarity are lost and cells gain a fibroblastic spindle-shape morphology which allow cells to infiltrate tissues and invade organs (Thiery, 2002). Interestingly, enforcing cav-1 expression in Panc 10.05 cells induced them to gain a different phenotype than control cells. Our cav-1 expressing cells displayed cell-cell adherens which were absent in the spindle-shape control cells. Then, we examined levels of E-cadherin as it is responsible for cell adherence and tight junction. Our immunoblotting and immunofluorescence analysis indicated that E-cadherin was absent in Panc10/pBabe but E-cadherin expression was restored after Panc 10.05 were infected with cav-1. Panc10/cav-1 displayed E-cadherin localization in the cell membrane. Restoration of E-cadherin expression is critical, as E-cadherin loss is correlated with undifferentiated and anaplastic pancreatic tumors leading to worse prognosis as compared to

differentiated pancreatic tumors that express E-cadherin (Winter et al., 2008). Moreover, recent studies implied that chemoresistant pancreatic cancer cells were deprived from E-cadherin, and to sensitize these cells to cytotoxic drugs, cells need to re-express E-cadherin (Arumugam et al., 2009). Similarly to E-cadherin function, we previously reported that cav-1 stabilizes β-catenin in the caveolae of the cell membrane (Galbiati et al., 2000). Such stabilization prevents β-catenin to form a complex with T-cell factor (TCF) which translocates to the nucleus to initiate EMT and invasion (Reichert, Muller, & Hunziker, 2000). The formation of this complex is also promoted by Snail, that has a reciprocal correlation with E-cadherin expression (Stemmer, de Craene, Berx, & Behrens, 2008; Q. Wang, Sun, Allgayer, & Yang, 2010). Snail suppresses E-cadherin expression through recruiting histone deacetylases (HDAC) binding to the E-boxes in the E-cadherin promoter (von Burstin et al., 2009). Also, Yin *et al.* showed that Snail overexpression in pancreatic cancer cells was correlated with lymph node invasion and distant metastasis (Yin et al., 2007). Also, they found that overexpressing Snail in Panc1 cells enhanced metastasis in orthotopic mice and promoted chemoresistance to 5-fluorouracil or gemcitabine. Our results show that cav-1 down-regulates Snail expression. To better understand how cav-1 affects

Snail expression, we investigated the three main molecules responsible for Snail activation, namely ERK, Smad2 and AKT, which are involved in phosphatidylinositol 3-kinase, mitogen-activated protein kinase (MAPK), TGF-β and (PI3K)/AKT pathways, respectively (Camara & Jarai, 2010; Grotegut, von Schweinitz, Christofori, & Lehembre, 2006; Larue & Bellacosa, 2005; Peinado, Quintanilla, & Cano, 2003). Li *et a.l* demonstrated that E-cadherin re-expression in breast cancer cells was not enough for EMT reversion unless ERK was suppressed (Q. Li & Mattingly, 2008). Moreover, previous studies showed that MAPK regulates TGF-β role to act as a tumor promoter (Chow et al., 2007). Also, TGF-β pathway activates by phosphorylation Smad2 to be translocated to the nucleus which bind with EMT transcriptional factors such ZEB1 or ZEB2 (K. A. Brown, Pietenpol, & Moses, 2007). Remarkably, our results show that cav-1 down-regulates both ERK and Smad2 activity restoring epithelial cell status. Another critical pathway down-regulated by cav-1 is AKT. AKT is not only an EMT inducer, but also it is a pro-survival and metastasis promoter molecule (Grille et al., 2003).

We also found that EMT features, such migration, invasion, and chemoresistance, were eventually altered. A previous study has demonstrated that cav-1, through ERK inhibition, suppresses migration and invasion in pancreatic cancer cells, which is confirmed in our study (F. Han, Gu, Chen, & Zhu, 2009). Importantly, cav-1 down-regulated AKT, which is a pro-survival molecule. Many previous studies indicated that AKT pathway in breast, lung, gastric, and uterine cancer is responsible for doxorubicin resistance (Gagnon, Van Themsche, Turner, Leblanc, & Asselin, 2008; Tari, Mehta, & Lopez-Berestein, 2001; Y. A. Wang, Johnson, Brown, & Dobson, 2009; Yu et al., 2008; Zhao et al., 2004). In this study, we also showed that cav-1 sensitize pancreatic cancer cell to doxorubicin through down-regulation of AKT pathway. Also, Arumugam *et al.* indicated that EMT induces chemoresistant toward gemcitabine and 5-flurouracil in pancreatic cancer cells (Arumugam et al., 2009), which is confirmed by our data that cav-1 inhibitions to EMT leads to cancer cell chemosensitization.

Conventional two-dimensional cell culture has been found to distort outcomes by changing cells environment from three-

dimensional environment to artificial flat rigid surfaces (Elsdale & Bard, 1972). On the contrary, three-dimensional cell culture system provides the most resembling environment for cells, where the cells sense the extracellular matrix and mediate various signals involving cell growth, migration, differentiation, survival (Cukierman, Pankov, & Yamada, 2002). Branching or tubulogenesis of cells in three-dimensional cultures requires EMT and activation of ERK pathway (Debnath & Brugge, 2005; Montesano, Matsumoto, Nakamura, & Orci, 1991; Soriano, Pepper, Nakamura, Orci, & Montesano, 1995). Here, we used three-dimensional culture to grow cav-1 expressing cells. Our data shows that Panc10/cav-1 cells displayed more significant regular spheres accompanied by less branching and irregularity of cell spheres after three days incubation with EGF. These results go along with Gutierrez-Barrera *et al.* results, where they showed that pancreatic cancer cell lines formed irregular spheres unlike the non cancerous human pancreatic ductal epithelial (HPDE) cell line (Gutierrez-Barrera, Menter, Abbruzzese, & Reddy, 2007).

Our laboratory have previously reported that cav-1 negatively regulates cell cycle and induces Inducing G_0/G_1 arrest

(Galbiati, Volonte et al., 2001). Here, we incubated cav-1 peptide containing the scaffolding domain of cav-1 with a metastatic cell line, Aspc-1 (Chen et al., 1982) for two days. Using flow cytometry, we found that Ap-CAv-1 also induced G_0/G_1 arrest and significantly reduces S and G_2/M phases. This result was also demonstrated by Han *et al.* in Panc-1 cells expressing cav-1 (F. Han, Gu, Chen, & Zhu, 2009).

Previous studies demonstrated cav-1 implication in growth tumor inhibition (F. Han, Gu, Chen, & Zhu, 2009; Zhang et al., 2008). Also, here, our results displayed cav-1 tumors were smaller in weight and volume compared to the Panc10/pBabe control tumors. More interestingly, cav-1 tumors showed nests of differentiated cells, which were absent in control tumors. These differentiated cells expressed high levels of E-cadherin and β-catenin in the plasma membrane and their expression were lost in poor differentiated cells. This remarkable finding suggests a critical role of cav-1in cell differentiation and epithelial cell plasticity.

Although our results describe cav-1 as a tumor suppressor in pancreatic cancer, clinical data portrays cav-1 as a tumor promoter and correlates it with tumor grade and poor prognosis (Witkiewicz et al., 2008). This different discrepancy is maybe due to the fact that cav-1 loses its tumor suppression role or gains an oncogenic function in higher tumor grades (Bonuccelli et al., 2009). Another possible explanation is that E-cadherin expression is a necessity for cav-1 to act as a tumor suppressor, as E-cadherin inhibits the β-catenin-TCF oncogenic pathway (Torres et al., 2007). This relation of cav-1 and E-cadherin complex seems to be important to determine the behavior of cav-1 which effects the overall behavior of pancreatic cancer cells.

In conclusion, our data demonstrate cav-1 as be a potential cure for pancreatic cancer. We showed that cav-1 retrieved epithelial status of pancreatic cells and maintained E-cadherin in plasma membrane and cell differentiation and polarity. These promising findings encourage us to expand our research to include other molecules and to study other possible mechanisms that will explain better cav-1 as a potential cure for pancreatic cancer.

7. Figures & Tables

Individual	Gene	Relative Risk	Risk by 70	Cancer Morphology	Other Cancers
No history	None	1	0.5%	NS	None
Breast Cancer	*BRCA2*	3.5-10X	5%	NS	Breast, ovary, and prostate
	BRCA1	2X	1%	Breast cancer with basaloid features	Breast, ovary, and prostate
FAMMM	*P16 (CDKN2A)*	20-34X	10%-17%	NS	Melanoma
Familial pancreatic cancer (3 FDR)	*Unknown*	32X	16%	NS	Unknown
Peutz-Jeghers	*STK11/LKB1*	132X	30%-60%	NS	Gastroesophageal, small bowel, colorectal, and breast
HNPCC	*hMHL1, hMSH2,* others	Unknown	< 5%	Medullary and colloid phenotypes	Colorectal, endometrial, stomach, ovarian, ureter and renal pelvis, biliary tract, and brain
Young-age-onset pancreatic cancer	*FANC-C* and *FANC-G*	Unknown	Unknown	NS	Unknown

Table.1: Genes associated with high risk of pancreatic cancer. 3 FDR, 3 or more first-degree relatives with pancreatic cancer; FAMMM, familial atypical multiple mole melanoma syndrome; HNPCC, hereditary nonpolyposis colorectal cancer syndrome; NS, nonspecific. Adopted with modification from Maitra *et al* (Maitra & Hruban, 2008).

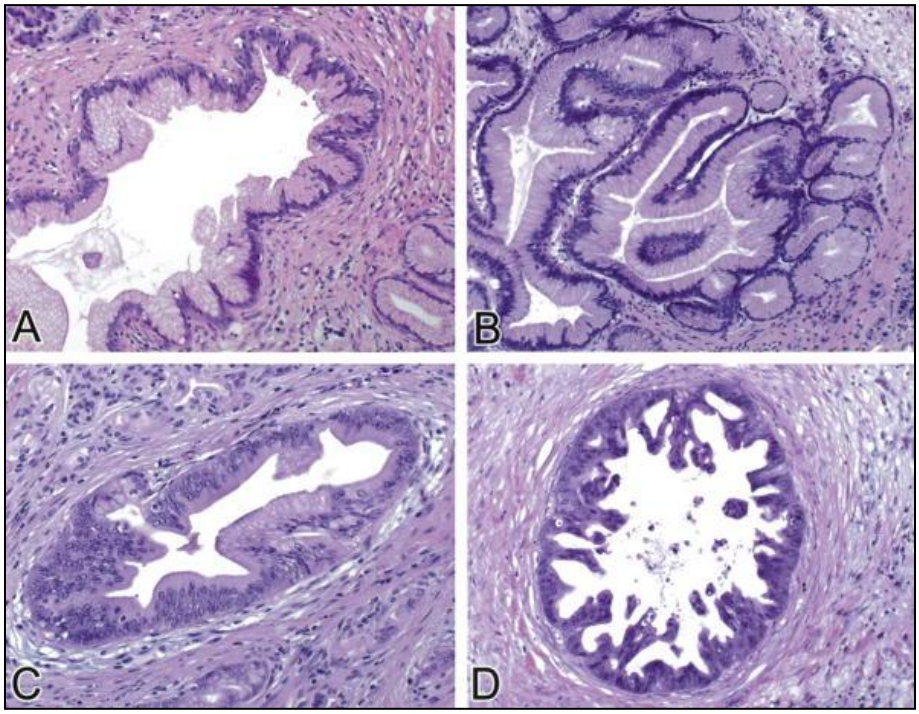


Figure.1: Examples of each grade of PanIN. In PanIN/L1A (A), the columnar cells have abundant apical cytoplasmic mucin but there are no papillae, the nuclei are basally located, and there is no cytologic atypia. In PanIN1B (B), similar cells line well-formed papillae. Ducts involved by PanIN2 (C) demonstrate full-thickness pseudostratification of nuclei with mild-to-moderate cytologic abnormalities. In PanIN3 (D), there is complete loss of polarity, budding of cellular tufts into the duct lumen, and significant nuclear atypia. Adopted from Hruban *et al* (R. H. Hruban et al., 2004).

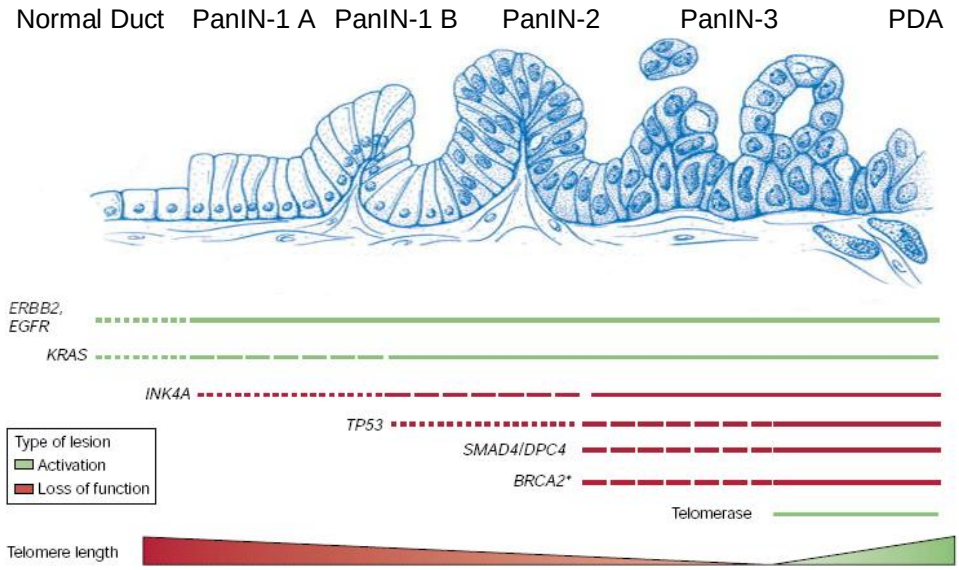
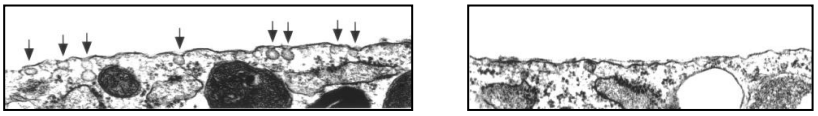


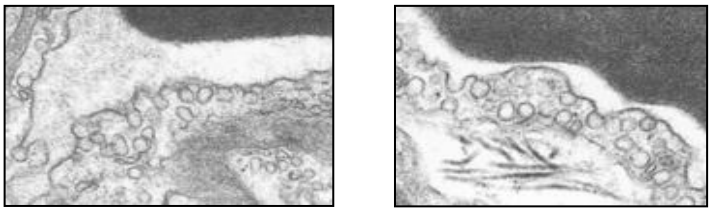
Figure.2: Pancreatic cancer progression. The upper blue part shows the progression of normal epithelial duct until the duct cells transform to pancreatic ductal adenocarcinoma –PDA- cells. The lower part shows the genetic modifications in pancreatic cancer progression, where it starts with telomeres shorting to prone the DNA to genetical mutations then activation mutation of *KRAS* in the first stages of cancer progression. Then several losses of tumor suppressor genes at the middle and late stages of cancer progression. The figure adopted with changes from Hruban *et al* (R. H. Hruban, Wilentz, & Kern, 2000).

MEFs



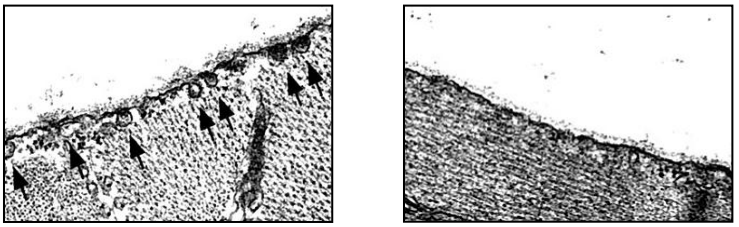
Wild-type Cav-1 KO

Lung Endothelial



Wild-type Cav-2 KO

Skeletal Muscle



Wild-type Cav-3 KO

Figure.3: Caveolae formation: cav-1 and cav-3 are required, but not cav-2 is required, for caveolae formation. Caveolae appear as 50- to 100 nm plasmalemmal invaginations (see arrows) by transmission electron microscopy. They are particularly abundant in mouse embryonic fibroblasts (MEFs), endothelial cells and skeletal muscle fibers. Note that in MEFs isolated from cav-1 null (-/-) mice (upper panel), the caveolae organelles are completely absent (Razani et al., 2001) and are also lacking in most other cells and tissues. Conversely, cav-2 is not required for caveolae formation since cav-2 null (-/-) mice do not display any perturbations of caveolae biogenesis (middle panel) (Razani, Wang et al., 2002). Cav-3 null (-/-) mice showed only defectiveness in striated muscle cell, such as skeletal muscle fibers (lowest panel) (Galbiati, Razani, & Lisanti, 2001b). Compiled from micrographs in (Galbiati, Razani, & Lisanti, 2001b; Razani et al., 2001; Razani, Wang et al., 2002)

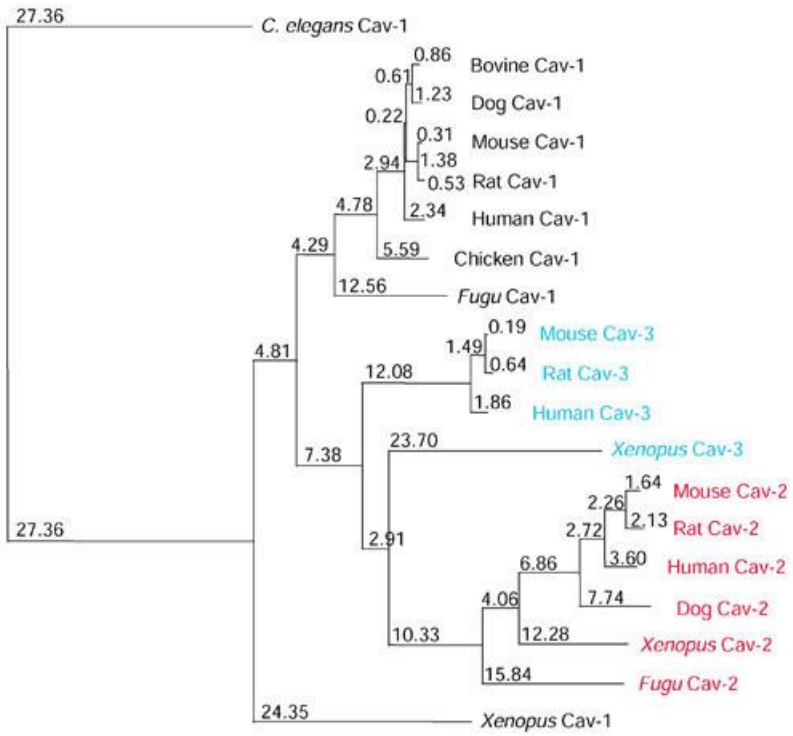


Figure.4: Phylogenic analysis of the caveolin gene family. Predicted evolutionary grouping for all caveolin family sequences. Adopted with change from Williams T *et al* (Williams & Lisanti, 2004).

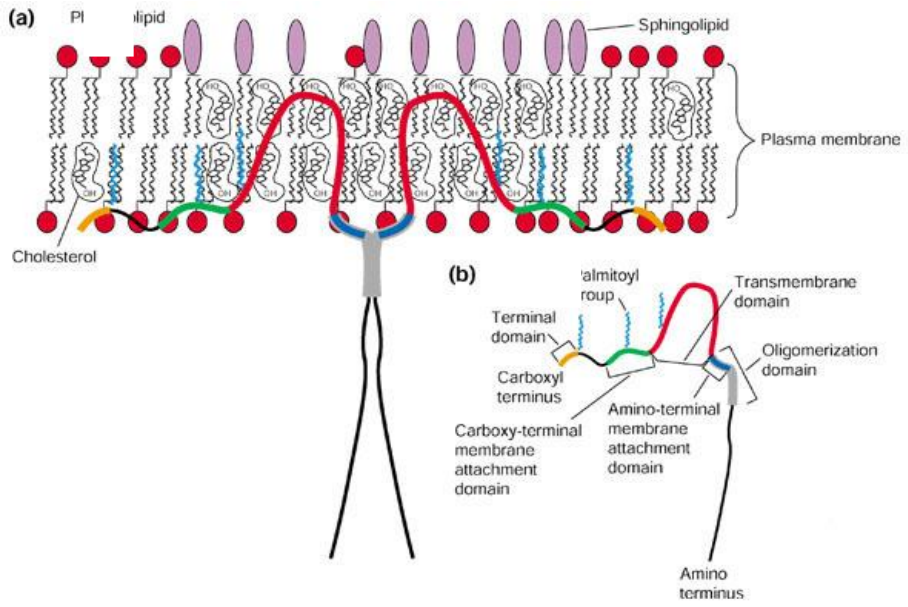


Figure.5: Predicted membrane topology of cav-1. Cav-1 monomer forms dimmers. However, ~14-16 monomers normally self-associate to form a single caveolin homo-oligomer (the caveolar assembly unit, akin to the clathrin triskelion). Both N- and C-terminal domains are oriented towards the Cytosolic face of the plasma membrane, with a hair-pin loop structure inserted with the membrane bilayers. The figure adopted from William *et al* (Williams & Lisanti, 2004).

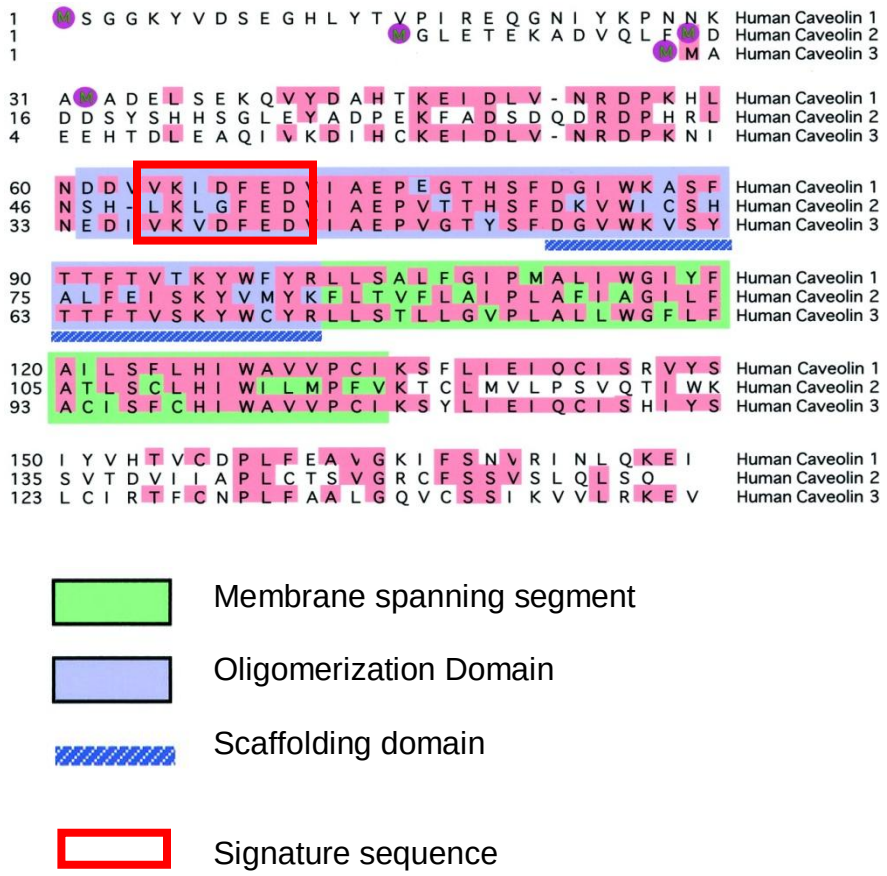


Figure.6: Protein sequence alignment of the human caveolin gene family. The red box indicates the “caveolin signature motif”. The oligomerization domain (Cav-1, residues 61-101), the scaffolding domain (Cav-1, residues 82-101), and the transmembrane region (Cav-1, residues 102-134) are all shown. Violet circules indicate translation initiation sites (methionine). The internal start site at position 32 in cav-1 indicates the start of the β-isoform of cav-1. Identical residues are high-lighten in rose. This figure is adopted with modification from Razani *et al* (Razani, Wang et al., 2002).

Gene	Species	Genbank Accession No.
Cav-1	Human	Z18951
	Bovine	U86639
	Dog	U47060
	Chicken	L01582
	Mouse	U07645
	Rat	AF439778
	Xenopus	AF455042
	Fugu	AJ010316
	C. elegans	U66405
Cav-2	Human	AF035752
	Dog	AF039223
	Mouse	AF141322
	Rat	AF439780
	Xenopus	AF455043
	Fugu	AJ010316
	C. elegans	U75587
Cav-3	Human	AF043101
	Mouse	U36579
	Rat	U31968
	Xenopus	AF455044

Tabel.2: List of cloned caveolin cDNAs.

Human Gene	Human Chromosomal Location	Exon	Size (bp)	Intron	Size (Kb)	Protein Residues Encoded	Length (AA)	% of similarity (identity) to Human Cav-1	% of similarity (identity) to Human Cav-2	Expression patterns
Cav-1	7q31.1	1 2 3	30 165 342	1 2	1.47 31.8	1-10 11-65 66-178	178	100	59 (40)	Ubiquitous; highest levels in adipocytes, endothelia, smooth muscle cells, and Type I pneumocytes
Cav-2	7q31.1	1 2a 2b	150 188 151	1 2	0.33 5.76	1-50 51-112 113-162	162	58 (38)	100	Co-expressed with Cav-1
Cav-3	3q25	1 2	114 342	1	?	1-38 39-151	151	85 (65)	60 (39)	Muscle-specific; primarily in skeletal and cardiac myocytes

Table.3: Overview of human caveolin genomic organization and protein product. Pb=base pair, kb=kilobases, AA= amino acids.

Oncogene	References
c-Myc	(Park et al., 2001; Timme et al., 2000)
N-Myc	(Park et al., 2001)
bcr-abl	(Koleske, Baltimore, & Lisanti, 1995)
v-Abl	(Engelman, Zhang, Razani, Pestell, & Lisanti, 1999; Koleske, Baltimore, & Lisanti, 1995)
H-Ras^{G12V}	(Engelman, Zhang, Razani, Pestell, & Lisanti, 1999; Koleske, Baltimore, & Lisanti, 1995)
H-Ras^{Q61L}	(Engelman, Zhang, Razani, Pestell, & Lisanti, 1999)
N-Ras^{Q61K}	(Engelman, Zhang, Razani, Pestell, & Lisanti, 1999)
K-Ras^{G12V}	(Engelman, Zhang, Razani, Pestell, & Lisanti, 1999)
c-Src or v-Src	(Engelman, Lee et al., 1998; Engelman, Zhang, Razani, Pestell, & Lisanti, 1999)
c-Neu/ErbB2	(Engelman, Lee et al., 1998)
crk1	(Koleske, Baltimore, & Lisanti, 1995)
HPV E6	(Razani et al., 2000)
PyMT Ag	(Koleske, Baltimore, & Lisanti, 1995)
v-Raf	(Engelman, Zhang, Razani, Pestell, & Lisanti, 1999)
PDK1	(Xie, Zeng, Waldman, & Glazer, 2003)

Tabel.4: Cellular oncoproteins that down-regulate Cav-1 expression.

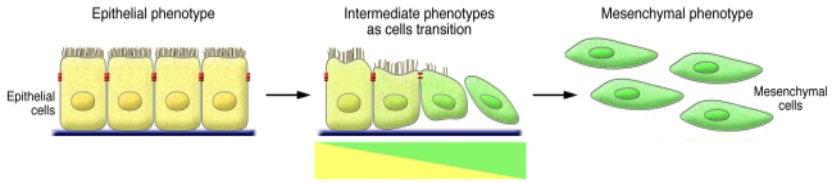


Figure.7: Epithelial-mesenchymal transition. Arranged epithelial cells show cells adherens, tight junction and desmosomes (Red junctions). Through epithelial-mesenchymal transition, epithelial cells transforms to mesenchymal phenotype, which is characterized by loss of orientation and high migration and invasion potential. Adopted with modification from Kalluri *et al* (Kalluri & Weinberg, 2009).

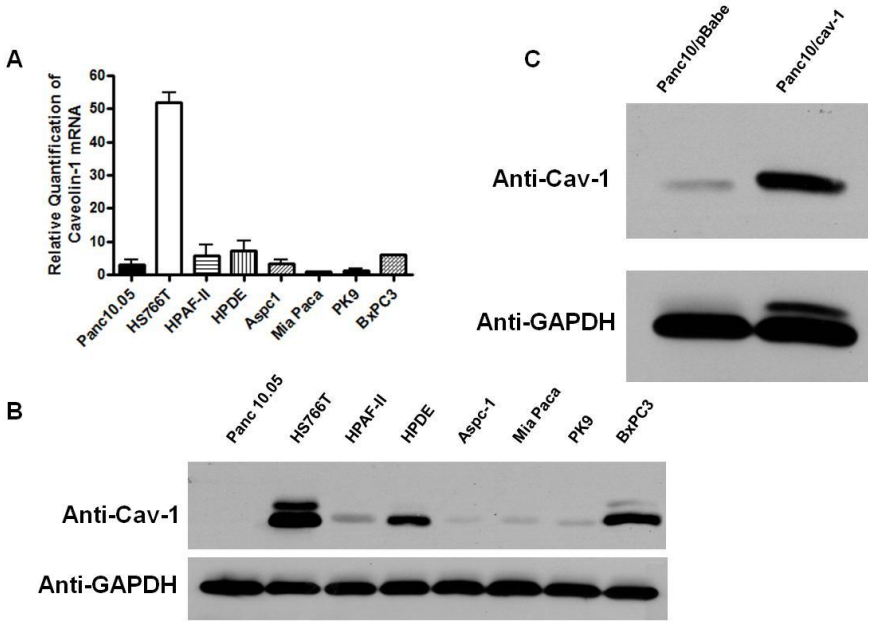


Figure.8: Caveolin-1 expression level in eight human pancreatic cell lines. *A*, eight pancreatic cell lines mRNA were extracted by Trizol. Using Real-time PCR, cav-1 mRNA was quantified and normalized with GAPDH. *B*, aliquots of the eight pancreatic cell lines containing 50 µg of total protein were separated with 12% SDS-PAGE then transferred to nitrocellulose membranes and immuneblotted with anti-cav-1 (N-20) against α isoform of caveolin-1 and reprobed with anti-GAPDH to confirm equal loading. *C,* successful infection of Panc 10.05 with pBabe (Empty vector) or with pBabe/cav-1.

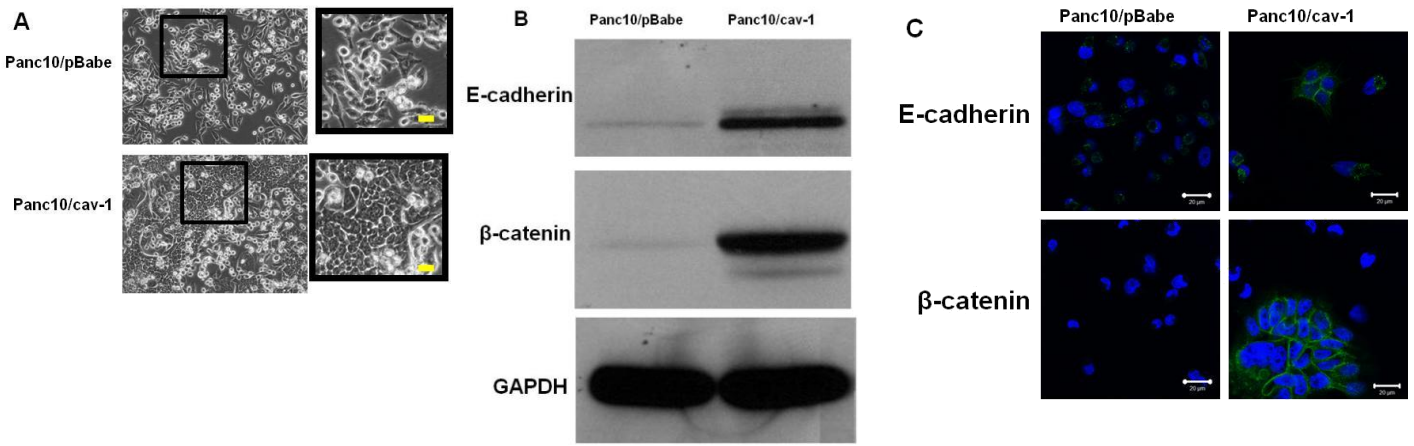


Figure.9: Cav-1 alters cells morphology and restores E-cadherin and β-catenin expression. *A,* Panc10/pBabe cells displays single spindle shape elongated cell, while Panc10/cav-1 show polygonal morphology and more cell-cell contact (10X). *Black box,* 8X zoom, *bar*, 20 µm. *B,* cav-1 expression restores E-cadherin and β-catenin. *C*, immunofluorescence analysis shows that, membranous E-cadherin and β-catenin signals were detected in Panc10/cav-1 cells, whereas these signals were lost in Panc10/pBabe cells. *Bar*, 20 µm.

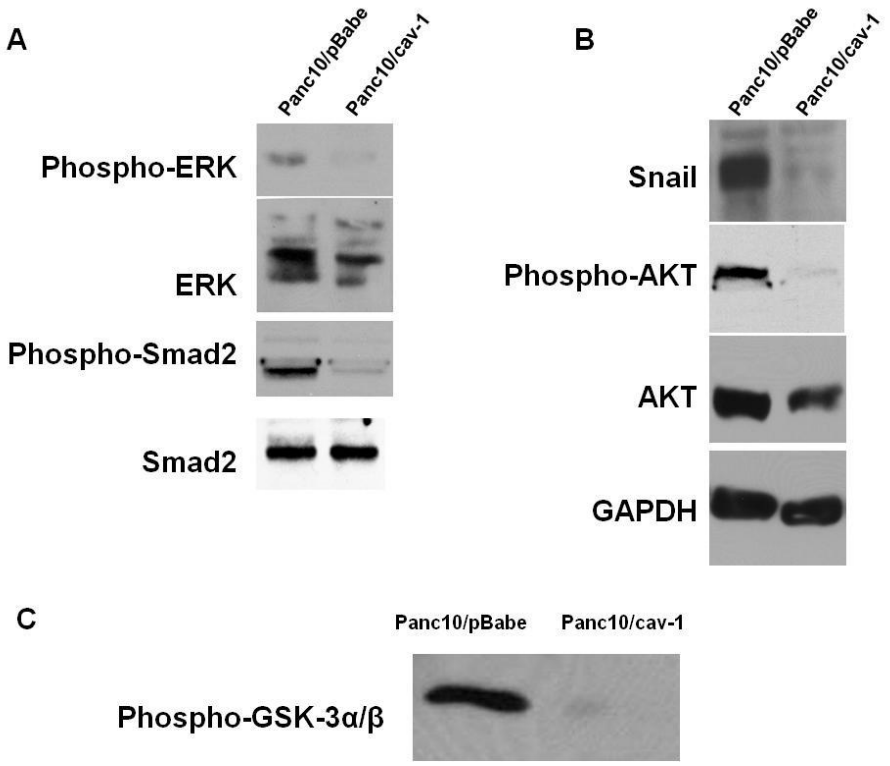


Figure.10: EMT pathways are downregulated by caveolin-1 expression. *A*,. phosphorylation levels of ERK and Smad2 were downregulated. *B,* total AKT, phospho-AKT and Snail expression were decreased in cav-1 expressing cells. *C*, AKT activity was downregulated in Panc10/cav-1. Fusion protein GSK-3*α/β* was used as a substrate for phospho-AKT. Note that AKT activity in cav-1 expressing cells is decreased compared to Panc10/pBabe.

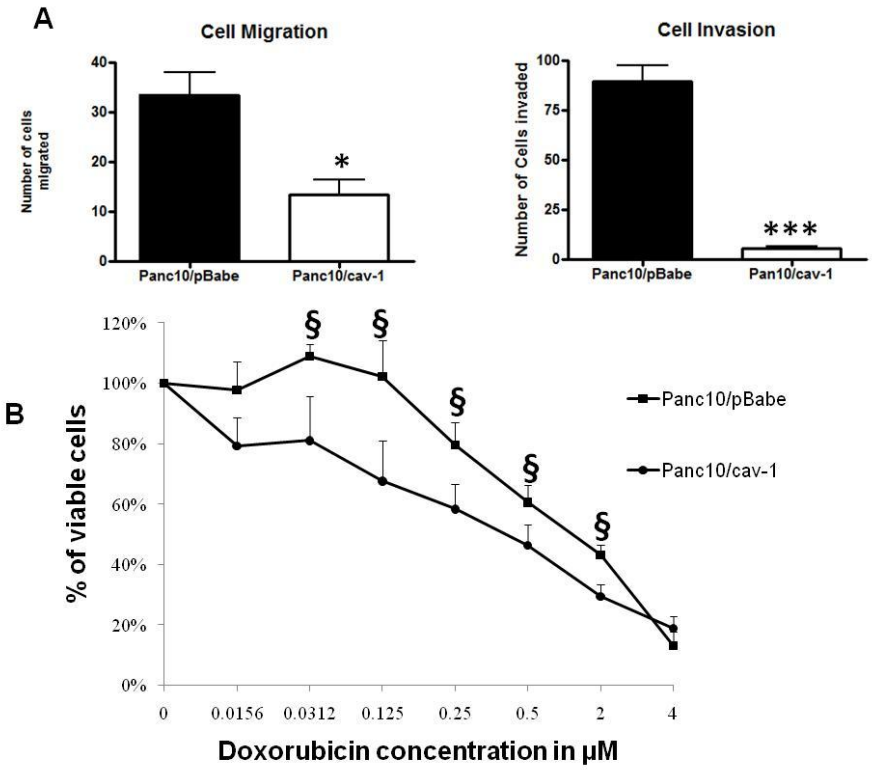


Figure.11: Cav-1 attenuates pancreatic cancer cells migration, invasion and drug resistance. *A,* migration and invasion are suppressed in cav-1 expressing cells. *Column,* average of cell number counted per field of three independent experiments, *bar,* SE. *B,* cav-1 sensitizes pancreatic cancer cells to doxorubicin. Each concentration was carried out in triplicates. *Bar,* SE. *, $P < 0.05$. §, $P < 0.01$, ***. $P < 0.0005$.

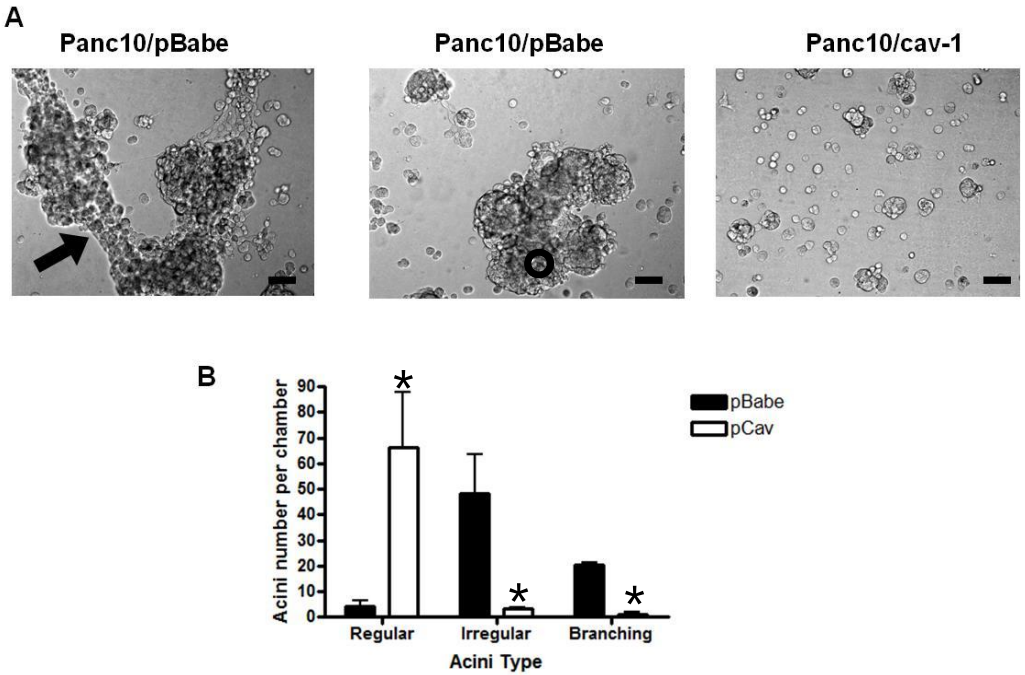


Figure.12: Cav-1 increases regularity of formed acini in three-dimensional Matrigel cultures. *A,* Cells were overlaid on 100% Matrigel basement membranes extract of reduced growth and incubated with complete growth medium and supplemented with 2% Matrigel and stimulated with 5 ng/ml EGF. Images acquisition was performed after 3 days of incubation. Panc10/cav-1 cells show small regular (smooth and spherical shape) acini, where Panc10/pBabe cells displayed branching (tubulogenesis) (Arrow) and more irregularity (irregular outline, multilobular) (O). *Bar,* 60 µm. *B,* Panc10/cav-1 cells showed more regular acini than Panc10/pBabe which were more irregular and branching acini. *Column,* every experiment was performed in quadruplicates, *bar,* SE. *, $P < 0.05$. ***, $P < 0.0005$.

Cell Line	G_0/G_1%	S%	G_2/M%	Apoptosis%
Aspc1+AP	58.525±1.96*	21.275±0.55*	18.475±0.64*	0.805±0.14
Aspc1+ 5mM AP-Cav-1	62.225±1.17	19.775±0.97	16.775±0.7	0.945±0.19

Tabel.5: Cave-1 down-regulate cell cycle. Flow cytometry analysis of Aspc-1 cells incubated with AP and AP-Cav-1 peptides to determine cell cycle and apoptosis. Data represent the mean ± SD of 4 independent experiments. *, $P < 0.05$.

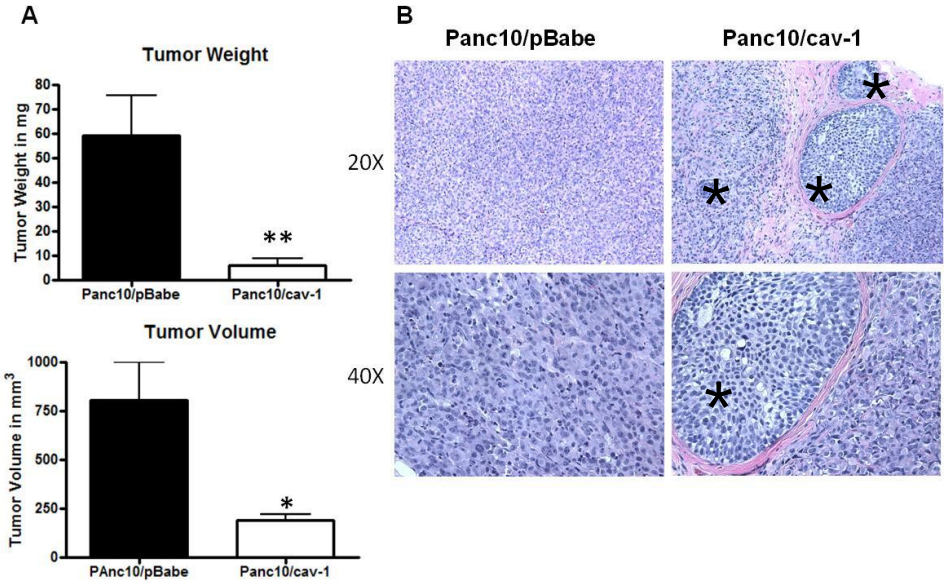


Figure.13: Cav-1 reduces tumor size and recovers tumors cells differentiation. *A,* the two histograms represent that cav-1 expression decreases tumors weight and volume. *Column,* mean of weight or volume of nine tumors for each cell line. *bar,* SE. *, $P < 0.01$. **, $P < 0.005$. *B*, cav-1 restores cancer cells differentiation. H&E staining shows islets of differentiated cells (Asterisk) only in Panc10/cav-1 tumors. Note that eosinophilic fibroblasts surrounds the differentiated cells.

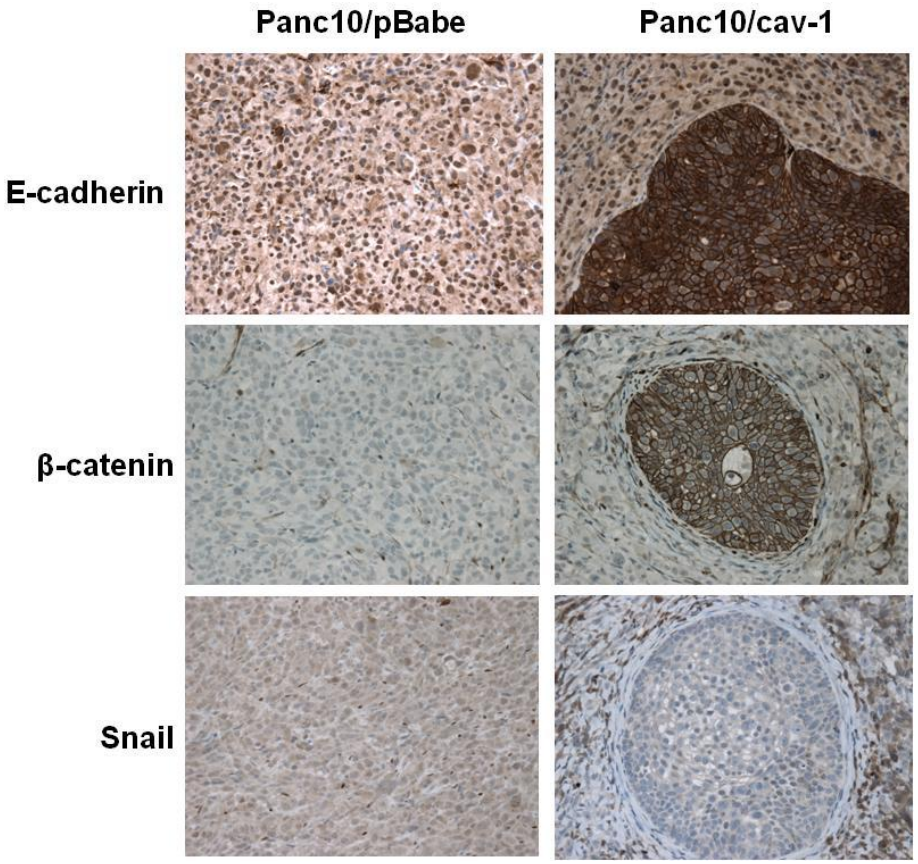


Figure.14: Cav-1 expression restores membranous E-cadherin in tumor differentiated islets. Differentiated cells in Panc10/cav-1 tumors express membranous E-cadherin and β-catenin as compared to Panc10/pBabe tumors. Note Snail expression in poor differentiated cells cytoplasm and nucleus, while Snail expression is suppressed in differentiated cells (40X).

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